Feeding behaviour and movement as sublethal endpoints to study the impact of pharmaceuticals on the freshwater amphipod *Gammarus pulex*

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Abstract

Pollution of the aquatic environment by pharmaceuticals is a well-established problem that has raised the attention of the scientific and public community since the late 1970s. However, there remain uncertainties about the possible adverse effects that pharmaceuticals compounds may have on non-target organisms.

The following thesis focuses on understanding the impact of pharmaceuticals on the behaviour of the freshwater amphipod *Gammarus pulex*. In particular, this thesis provides an evaluation of the effectiveness and applicability of *G. pulex* feeding behaviour as a sublethal endpoint in ecotoxicology.

G. pulex plays an important role in the decomposition of organic matter in lotic environments. *Gammarus* spp. feeding behaviour has often been studied as a sublethal endpoint in ecotoxicology. Currently, there is no official standardised methodology and differences in the method can be encountered in several steps of the experimental design: acclimation phase, food preparation and feeding rate calculation. The lack of standardisation for feeding studies can affect the outcome of an experiment and weaken possible comparisons between published literature.

The comparability and applicability of five different feeding equations were determined over periods of 24 h and 7 days, and the toxicity of the antidiabetic drug metformin was also investigated. One of the tested feeding equations was found to produce results that did not reflect *G. pulex* feeding activity and cannot be considered equivalent to the others. *G. pulex* feeding rate was inhibited after 2 days by a concentration of 10 µg/L of metformin compared to the control, whereas the swimming velocity was not altered. These results further support the necessity of developing a standard feeding assay for Gammarids, but also highlight the ecological impact that metformin might have in freshwater environments.

The indirect impact on *G. pulex* feeding behaviour of a mixture of antibiotics (sulfamethoxazole and trimethoprim) was assessed. Fungal biomass on the

leaves surface and bacterial abundance in the conditioning water were also measured. *G. pulex* specimens ate significantly less when they were provided with leaf discs that were conditioned in a mixture of the two antibiotics, each at a concentration of 2 and 20 μg/L. No differences were measured in the fungal biomass, whereas bacterial abundance was significantly lower in the presence of the 20 μg/l and 200 μg/L mixture concentrations. The tested mixture could indirectly affect *G. pulex* feeding behaviour and reduce the bacterial abundance in water, but this was only measured at concentrations that are typical of heavily polluted streams (2 μg/L) or are at least 10 times higher (20 μg/L) than the highest reported concentration in the environment.

Behavioural analyses, in terms of movement, ventilation, feeding rate and swimming velocity, were determined after exposure to three concentrations of the antidepressant, venlafaxine. Over a period of 11 days, organisms exposed to 0.02 μg/l were found to move significantly more compared to the control, whereas ventilation was not affected. *G. pulex* feeding activity was found to significantly increased after 24 h and after 2-day exposure to 20 μg/l venlafaxine concentration. A significant increase in swimming velocity was measure after 7 days in organisms that were exposed to 20 μg/l of venlafaxine. Collectively, these results may have implications for the ecological success of *G. pulex*.

Overall, this research has demonstrated that *G. pulex* feeding behaviour is a sensitive sublethal endpoint. *G. pulex* feeding activity was affected by exposure to metformin and venlafaxine, and by indirect exposure to the antibiotic mixture of sulfamethoxazole and trimethoprim. A standardised methodology would allow comparisons between studies and the possibility of feeding behaviour to be included in environmental risk assessments. The current study has also shown how different pharmaceuticals can induce alterations in different behavioural endpoints (movement, swimming velocity, feeding rate) in *G. pulex.*

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Whilst being registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Equations from Chapter 3:

(Equation 1) $$FR = \frac{A_i*(CF_A) - A_f}{w*t}$$

(Equation a) $$CF_A = \frac{\left[\sum\left(\frac{A_{cf}}{A_{ci}}\right)\right]}{n}$$

(Equation 2) $$FR = \frac{A_i - A_f}{w*t}$$

(Equation 3) $$FR = \frac{L_i - \frac{L_f}{CF_1}}{w*t}$$

(Equation 4) $$FR = \frac{L_i*(CF_1) - L_f}{w*t}$$

(Equation 5) $$FR = \frac{L_i*(CF_2) - L_f}{w*t}$$

(Equation b) $$CF_1 = \frac{\left[\sum\left(\frac{C_f}{C_i}\right)\right]}{n}$$

(Equation c) $$CF_2 = \frac{\sum\left[\frac{C_i - C_f}{C_i}\right]}{n}$$

Equations from Chapter 4 and 5:

$$Bacteria\ per\ mL = cell\ count * \frac{1000}{Flow\ rate * time\ (\mathrm{s})} * Dilution\ Factor$$

$$FR = \frac{A_i*(CF_A) - A_f}{w*t}$$

$$CF_A = \frac{\left[\sum\left(\frac{A_{cf}}{A_{ci}}\right)\right]}{n}$$

$$FR = \frac{L_i*(CF_1) - L_f}{w*t}$$

$$CF_1 = \frac{\left[\sum\left(\frac{C_f}{C_i}\right)\right]}{n}$$

List of abbreviations

AFDW: Ash-free dry weight

AMPK: AMP kinase

AOPs: Advanced oxidation processes

BLD: Below limit of detection

CAS: conventional activated sludge

CF: Leaf change correction factor

CHH: crustacean hyperglycaemic hormone

CIP: Chemical investigation program

CPOM: coarse particulate organic matter

ECs : Emerging contaminants

EDC: endocrine disrupting compound

EMA: European Medicines Agency

EMEA: European Agency for Evaluation of Medicinal Products

ERA: Environmental Risk assessment

FBR: Fixed bed bioreactors

FPOM: fine particulate organic matter

FR: feeding rate

LC_{50}: median lethal dose

LOEC: lowest observed effect concentration

MBBR: moving bed biofilm reactors

MBR: membrane Bioreactors

MET: metformin

MFB: Multispecies Freshwater Biomonitor

MOA: Mode of action

N.d.: not detected

OCT: oxytetracycline

OECD: organisation for economic cooperation and development

PEC: Predicted Environmental concentration

PNEC: Predicted no-effect concentration

PPCPs: Pharmaceuticals and Personal Care products

PUFA: polyunsaturated fatty acids

QIC: quadruple impedance conversion

SDZ: sulfadiazine

SMX: sulfamethoxazole

SNRIs: Serotonin and norepinephrine reuptake inhibitors

SSRIs: selective serotonin reuptake inhibitors

TMP: trimethoprim

VEN: venlafaxine

WFD: water framework directive

WWTPs: Wastewater Treatment Plants

Dissemination

Published publication[1]:

Consolandi, G., Ford, A.T. & Bloor, M.C. (2019). Feeding behavioural studies with freshwater Gammarus spp.: the importance of a standardised methodology. In: *Reviews of Environmental Contamination and Toxicology (Continuation of Residues Reviews).*

Conference abstract[2]:

<u>Platform presentation</u>:

"Impact of antibiotics on the feeding rate of the freshwater shrimp *Gammarus pulex.*"

Presented at SETAC (Society of Environmental Toxicology and Chemistry) Europe 28th Annual meeting in Rome, May 13th-17th , 2018.

<u>Poster presentations</u>:

"Impact of antibiotics on the feeding rate of the freshwater shrimp *Gammarus pulex.*"

Presented at SETAC (Society of Environmental Toxicology and Chemistry) Europe 28th Annual meeting in Rome, May 13th-17th , 2018.

"Using the Multispecies Freshwater Biomonitor to assess the potential impact of the antidepressant venlafaxine on the amphipod *Gammarus pulex*."

Presented at SETAC (Society of Environmental Toxicology and Chemistry) Europe 29th Annual meeting in Helsinki, May 26th-30th, 2019.

"Effects of the antidepressant venlafaxine on the feeding rate and behaviour of the freshwater amphipod *Gammarus pulex*."

[1] The published manuscript can be found in Appendix A
[2] A compilation of conference abstracts and certificates can be found in Appendix B

Presented at SETAC (Society of Environmental Toxicology and Chemistry) Europe 29th Annual meeting in Helsinki, May 26th-30th, 2019.

Chapter 1: Introduction

1.1 Emerging contaminants

Since the first report of pharmaceuticals compounds in the environment during the late 1970s and early 1980s (Hignite & Azarnoff, 1977; Richardson & Bowron, 1985), environmental scientists and the public community have shown an increased interest and concern about the source and fate of new generation pollutants, nowadays known as Emerging Contaminants (ECs) (Oberg & Leopold, 2019) (Figure 1.1). The acronym ECs is used to describe compounds of anthropogenic origin, such as plastics, nanomaterials, illegal drugs, pesticides, surfactants, pharmaceuticals and personal care products (PPCPs) (Taheran et al., 2018). Emerging chemicals have been defined as "*a chemical for which there are increasing concerns regarding its potential risks to humans and ecological systems, including endocrine disruption and neurotoxicity"* (Diamond et al., 2011). Even though ECs have been detected around the world in many different environmental systems (e.g. coastal areas, surface waters, groundwater, soil, wastewater effluent and in drinking water) (Wilkinson et al., 2017), they are still poorly regulated and consequently they are continually discharged into the environment, where their potential effects are still mainly unknown (Daughton, 2001).

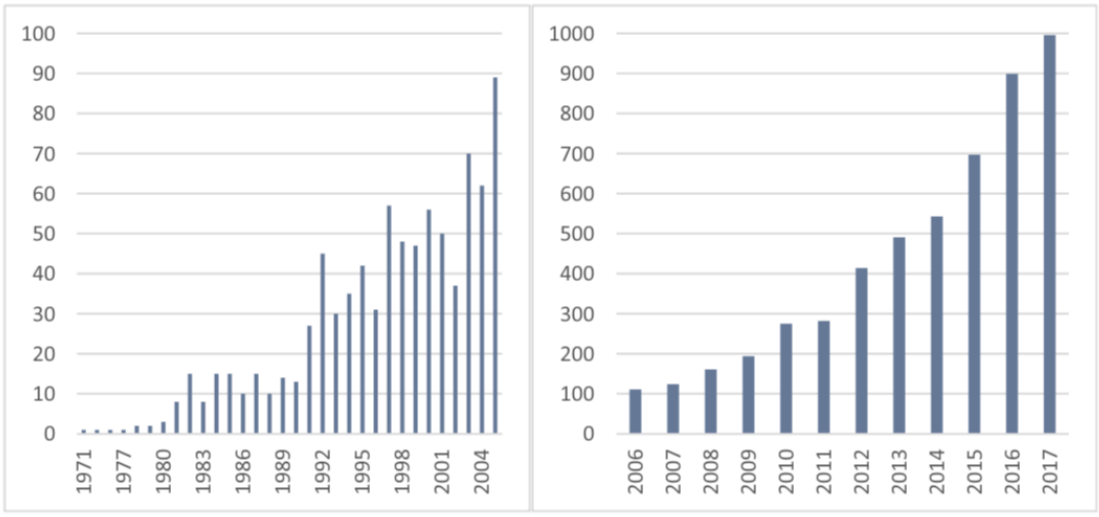

Figure 1.1. Number of publications per year dealing with micropollutants or emerging contaminants/pollutants (A) from 1971 to 2005 and (B) from 2006 to 2017 (Oberg & Leopold, 2019).

Even though ECs are consumed and discharged worldwide, they are not necessarily considered high production chemicals (Ankley et al., 2007). Consequently, they are not commonly monitored on a regular basis, and because of the lack of relevant information regarding their impact at low concentrations, it is challenging for governments and policy makers to regulate them.

It is only in recent years that the Guidelines on Environmental Risk Assessment of Medicinal Products for Human Use (EMEA, 2006) and new European Directives (e.g. Directive 2000/60/EC, Directive 2001/83/EC, Directive 2008/105/EC, Decision 2015/495/EU and Decision 2018/840/EU) have been issued, with the aims of protecting and restoring water bodies as well as limiting and monitoring certain contaminants, identified as priority pollutants (see Chapter 1, section 1.6).

1.2 Pharmaceuticals and Personal Care Products (PPCPs)

Pharmaceuticals and personal care products (PPCPs) is a wide class of contaminants that includes many different chemicals, such as illicit drugs, medications, lotions, make-up products, toothpastes, deodorants, perfumes, disinfectants, fragrances, shampoos and sun creams (Boxall et al., 2012; Brausch & Rand, 2011; Ebele et al., 2017). The main difference between pharmaceuticals and personal care products is the way that the substances are used. Personal care products are commonly applied externally on the body and their chemical structure remains unaltered, whereas pharmaceuticals are usually designed to be ingested and undergo metabolic reactions in order to perform their therapeutic function (Birch et al., 2015). Nonetheless, PPCPs are included in a single group, because despite having different modes of action, these contaminants can reach the environment through common routes (e.g. waste water and improper disposal), they are composed of biologically active molecules and are designed to work at low concentrations.

1.3 Pharmaceuticals in the aquatic environment

Pharmaceutical residues have been detected in many different aquatic systems in the ng/L and µg/L range (Carballa et al., 2004; Ebele et al., 2017;

Gaw et al., 2014; Hughes et al., 2012; Küster & Adler, 2014; Nebot et al., 2015; Petrie et al., 2014; Santos et al., 2010), (Table 1.1). Precisely because pharmaceuticals are designed to work at low concentrations, they may induce effects in non-target organisms with similar metabolic or enzymatic pathways (Ebele et al., 2017; Santos et al., 2010) and they may affect functions such as growth, reproduction and development (Fabbri & Franzellitti, 2016).

Due to their worldwide exponential consumption, pharmaceuticals are constantly and perpetually discharged into natural water bodies (Couto et al., 2019; Mezzelani et al., 2018) . Moreover, their utilisation and consequent environmental concentrations are increasing on an annual basis as a result of different factors, such as the expanding number of medications available, the increasing accessibility to medicines worldwide, affordability and population growth (Comber et al., 2018). Even though their half-lives are usually considerably shorter than other pollutants' (e.g. DDT, PCBs) (Ankley et al., 2007), they are nowadays considered pseudo-persistent pollutants, as their half-lives are basically surpassed by the input rates from wastewater effluents (Daughton, 2002). Consequently, non-target organisms are chronically exposed to sublethal doses of pharmaceuticals (Fent et al., 2006). However there is still lack of knowledge regarding the effects of longterm exposure to these emerging pollutants (Cunningham et al., 2006) and especially regarding the potential synergistic interactions (Backhaus & Faust, 2012). Indeed, pollutants do not occur singularly in the environment, but as complex mixtures (Vasquez et al., 2014). Pharmaceuticals may interact within each other, but also with their metabolites. They may produce combined effects through independent non-interactive or through interactive interactions (Gonzalez-Rey et al., 2014).

This constant ongoing release of pharmaceutical compounds is not only a prospective risk for the environment, but also a potential hazard for public health (Briones et al., 2016; Ebele et al., 2017). For example, in the last few years pharmaceuticals and derived compounds have also been detected in drinking water (Deblonde et al., 2011; Ternes, 1998; Touraud et al., 2011). Organisms may bioaccumulate these compounds and consequently, pass them on to humans (Puckowski et al., 2016) through biomagnification. Furthermore,

pharmaceuticals, such as antibiotics could lead to development of antibiotic resistant genes in those microorganisms that are constantly exposed to sublethal antibiotic concentrations (Gaw et al., 2014).

Table 1.1. Concentrations of different pharmaceuticals in different aquatic compartments. Abbreviations: BLD: below limit of detection; n.d.: not detected.

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Acetaminophen	anti-inflammatory		20.8		River upstream	South Africa	Archer et al., 2017
			63.7		River downstream	South Africa	Archer et al., 2017
		5900	18000	150000	Raw influent	USA	Blair et al., 2013b
		<BLD	39	650	Final effluent	USA	Blair et al., 2013b
Amitriptyline	antidepressant	<BLD		<BLD	River	United Kingdom	Kasprzyk-Hodern et al., 2007
		3		21	River	United Kingdom	Kasprzyk-Hodern et al., 2008
Amoxycillin	antibiotic	39		245	River	United Kingdom	Kasprzyk-Hodern et al., 2007
		10		622	River	United Kingdom	Kasprzyk-Hodern et al., 2008
			1400	6940	Influent	Australia	Watkinson et al., 2009
			n.d.	50	Effluent	Australia	Watkinson et al., 2009
			n.d.	200	Surface water	Australia	Watkinson et al., 2009
			n.d.	n.d.	Drinking water	Australia	Watkinson et al., 2009
Atenolol	beta-blocker		156.2		River upstream	South Africa	Archer et al., 2017
			272		River downstream	South Africa	Archer et al., 2017
Carbamazepine	anti-epileptic	4		9	River	United Kingdom	Kasprzyk-Hodern et al., 2007
		5		356	River	United Kingdom	Kasprzyk-Hodern et al., 2008
		20		22.3	Tap water	Portugal	Paíga et al., 2017

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Carbamazepine		n.d.		22.1	Bottled water	Portugal	Paíga et al., 2017
		32.9		34.4	River	Portugal	Paíga et al., 2017
		66.2		110.9	Influent	Portugal	Paíga et al., 2017
		98.5		244.9	Effluent	Portugal	Paíga et al., 2017
				1680	Influent	Sweden	Bendz et al., 2005
				1180	Effluent	Sweden	Bendz et al., 2005
		43		127	Influent	South Korea	Behera et al., 2011
		40		74	Effluent	South Korea	Behera et al., 2011
			157.1		River upstream	South Africa	Archer et al., 2017
			279.5		River downstream	South Africa	Archer et al., 2017
		21	72	310	Raw influent	USA	Blair et al., 2013b
		27	180	340	Final effluent	USA	Blair et al., 2013b
			220		Influent	Netherlands	Oosterhuis et al., 2013
			200		Effluent	Netherlands	Oosterhuis et al., 2013
Ciprofloxacin	antibiotic	n.d.		n.d.	Tap water	Portugal	Paíga et al., 2017
		n.d.		n.d.	Bottled water	Portugal	Paíga et al., 2017
		n.d.		n.d.	River	Portugal	Paíga et al., 2017
		n.d.		118.9	Influent	Portugal	Paíga et al., 2017
		n.d.		96.6	Effluent	Portugal	Paíga et al., 2017
				740	Surface water	Spain	Gracia-Lor et al., 2011
				2292	Effluent	Spain	Gracia-Lor et al., 2011
		<BLD	<BLD	87	Raw influent	USA	Blair et al., 2013b
		<BLD	<BLD	<BLD	Final effluent	USA	Blair et al., 2013b

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Ciprofloxacin			600	1100	Influent	Australia	Watkinson et al., 2009
			n.d.	1300	Surface water	Australia	Watkinson et al., 2009
			n.d.	n.d.	Drinking water	Australia	Watkinson et al., 2009
Clarithromycin	antibiotic		76.2		River upstream	South Africa	Archer et al., 2017
			235.5		River downstream	South Africa	Archer et al., 2017
		<BLD	<BLD	5.6	Raw influent	USA	Blair et al., 2013b
		<BLD	<BLD	19	Final effluent	USA	Blair et al., 2013b
Diclofenac	anti-inflammatory	1		85	River	United Kingdom	Kasprzyk-Hodern et al., 2008
				160	Influent	Sweden	Bendz et al., 2005
				120	Effluent	Sweden	Bendz et al., 2005
				17	River	Malaysia	Al-Odaini et al., 2010
				217	Effluent	Malaysia	Al-Odaini et al., 2010
				1490	Influent	Spain	Gracia-Lor et al., 2010
				740	Effluent	Spain	Gracia-Lor et al., 2010
				358	Surface water	Spain	Gracia-Lor et al., 2011
				690	Effluent	Spain	Gracia-Lor et al., 2011
		<20		<20	River upstream	United Kingdom	Ashton et al., 2004
		<20		568	River downstream	United Kingdom	Ashton et al., 2004
		<20		2349	Effluent	United Kingdom	Ashton et al., 2004
		59		243	Influent	South Korea	Behera et al., 2011

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Diclofenac		13		49	Effluent	South Korea	Behera et al., 2011
		2		43	Influent	Luxembourg	Pailler et al., 2009
		<BLD		78	Effluent	Luxembourg	Pailler et al., 2009
		<BLD		55	River	Luxembourg	Pailler et al., 2009
		<BLD		19	River	Luxembourg	Pailler et al., 2009
				2838	Effluent	United Kingdom	Kay et al., 2017
				2991	Receiving water	United Kingdom	Kay et al., 2017
			467.4		River upstream	South Africa	Archer et al., 2017
			1461.5		River downstream	South Africa	Archer et al., 2017
			340		Influent	Netherlands	Oosterhuis et al., 2013
			200		Effluent	Netherlands	Oosterhuis et al., 2013
Erythromycin-H_2O	antibiotic	7		22	River	United Kingdom	Kasprzyk-Hodern et al., 2007
				78	Surface water	Spain	Gracia-Lor et al., 2011
				82	Effluent	Spain	Gracia-Lor et al., 2011
		<10		57	River upstream	United Kingdom	Ashton et al., 2004
		<10		1022	River downstream	United Kingdom	Ashton et al., 2004
		<10		1842	Effluent	United Kingdom	Ashton et al., 2004
				1857	Effluent	United Kingdom	Kay et al., 2017

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Erythromycin-H_2O				1378	Receiving water	United Kingdom	Kay et al., 2017
Fluoxetine	antidepressant	n.d.		1.90	Tap water	Portugal	Paíga et al., 2017
		n.d.		0.27	Bottled water	Portugal	Paíga et al., 2017
		3.3		3.7	River	Portugal	Paíga et al., 2017
		5.2		8.8	Influent	Portugal	Paíga et al., 2017
		12.9		27.5	Effluent	Portugal	Paíga et al., 2017
		20		91	Raw wastewater	Canada	Metcalfe et al., 2010
		1.6		43.2	River	USA	Schultz et al., 2010
		0.5		29	River	USA	Schultz et al., 2010
			34.4		River upstream	South Africa	Archer et al., 2017
			109.2		River downstream	South Africa	Archer et al., 2017
		6.1	20	95	Raw influent	USA	Blair et al., 2013b
		<BLD	28	96	Final effluent	USA	Blair et al., 2013b
Gemfibrozil	antihyperlipidemic	101		318	Influent	South Korea	Behera et al., 2011
		9		26	Effluent	South Korea	Behera et al., 2011
		29	180	1200	Raw influent	USA	Blair et al., 2013b
		30	170	1100	Final effluent	USA	Blair et al., 2013b
Ibuprofen	anti-inflammatory	4		100	River	United Kingdom	Kasprzyk-Hodern et al., 2008
		<BLD		<BLD	Tap water	Portugal	Paíga et al., 2017
		<BLD		<BLD	Bottled water	Portugal	Paíga et al., 2017
		<BLD		<BLD	River	Portugal	Paíga et al., 2017
		4389.3		14124.8	Influent	Portugal	Paíga et al., 2017

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Ibuprofen		323.7		517.4	Effluent	Portugal	Paíga et al., 2017
				3590	Influent	Sweden	Bendz et al., 2005
				150	Effluent	Sweden	Bendz et al., 2005
				39800	Influent	Spain	Gracia-Lor et al., 2010
				<BLD	Effluent	Spain	Gracia-Lor et al., 2010
				2850	Surface water	Spain	Gracia-Lor et al., 2011
				15100	Effluent	Spain	Gracia-Lor et al., 2011
		<20		1555	River upstream	United Kingdom	Ashton et al., 2004
		<20		5044	River downstream	United Kingdom	Ashton et al., 2004
		<20		27256	Effluent	United Kingdom	Ashton et al., 2004
		1599		2843	Influent	South Korea	Behera et al., 2011
		15		75	Effluent	South Korea	Behera et al., 2011
		82		3080	Influent	Luxembourg	Pailler et al., 2009
		3		359	Effluent	Luxembourg	Pailler et al., 2009
		10		295	River	Luxembourg	Pailler et al., 2009
		9		2983	River	Luxembourg	Pailler et al., 2009
				4617	Effluent	United Kingdom	Kay et al., 2017
				4838	Receiving water	United Kingdom	Kay et al., 2017
			153.3		River upstream	South Africa	Archer et al., 2017
			312.1		River downstream	South Africa	Archer et al., 2017

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Ibuprofen		670	2100	11000	Raw influent	USA	Blair et al., 2013b
		<BLD	<BLD	<BLD	Final effluent	USA	Blair et al., 2013b
Ketoprofen	anti-inflammatory		642.2		River upstream	South Africa	Archer et al., 2017
			330.3		River downstream	South Africa	Archer et al., 2017
Metformin	antidiabetic	86000		142300	Influent	Germany	Trautwein et al., 2014
		6400		3400	Effluent	Germany	Trautwein et al., 2014
				216	River	Germany	Trautwein et al., 2014
		35		150	Lake	Germany	Trautwein et al., 2014
				293	River	Malaysia	Al-Odaini et al., 2010
				16	Effluent	Malaysia	Al-Odaini et al., 2010
			73.3		River upstream	South Africa	Archer et al., 2017
			174.6		River downstream	South Africa	Archer et al., 2017
		3200	55000	100000	Raw influent	USA	Blair et al., 2013b
		640	26000	47000	Final effluent	USA	Blair et al., 2013b
		103	174	249	Influent	Greece	Kosma et al., 2015
		<BLD	<BLD	23	Effluent	Greece	Kosma et al., 2015
			73730		Influent	Netherlands	Oosterhuis et al., 2013
			1820		Effluent	Netherlands	Oosterhuis et al., 2013
			105000		Influent	Germany	Scheurer et al., 2012
			2700		Effluent	Germany	Scheurer et al., 2012
			3100		River	Germany	Scheurer et al., 2012
Paracetamol	anti-inflammatory	216		1388	River	United Kingdom	Kasprzyk-Hodern et al., 2007

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Paracetamol		36		1188	River	United Kingdom	Kasprzyk-Hodern et al., 2008
Propranolol	non-selective beta-blocker			146	Effluent	United Kingdom	Kay et al., 2017
				165	Receiving water	United Kingdom	Kay et al., 2017
Sulfamethoxazole	antibiotic	<BLD		<BLD	River	United Kingdom	Kasprzyk-Hodern et al., 2007
		<0.5		2	River	United Kingdom	Kasprzyk-Hodern et al., 2008
		n.d.		n.d.	Tap water	Portugal	Paíga et al., 2017
		n.d.		n.d.	Bottled water	Portugal	Paíga et al., 2017
		n.d.		n.d.	River	Portugal	Paíga et al., 2017
		n.d.		224.1	Influent	Portugal	Paíga et al., 2017
		n.d.		73.4	Effluent	Portugal	Paíga et al., 2017
				20	Influent	Sweden	Bendz et al., 2005
				70	Effluent	Sweden	Bendz et al., 2005
				33	Surface water	Spain	Gracia-Lor et al., 2011
				432	Effluent	Spain	Gracia-Lor et al., 2011
		<50		<50	River upstream	United Kingdom	Ashton et al., 2004
		<50		<50	River downstream	United Kingdom	Ashton et al., 2004
		<50		132	Effluent	United Kingdom	Ashton et al., 2004
		79		216	Influent	South Korea	Behera et al., 2011

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Sulfamethoxazole		20		162	Effluent	South Korea	Behera et al., 2011
		13		155	Influent	Luxembourg	Pailler et al., 2009
		4		39	Effluent	Luxembourg	Pailler et al., 2009
		1		22	River	Luxembourg	Pailler et al., 2009
		<BLD		5	River	Luxembourg	Pailler et al., 2009
			757.4		River upstream	South Africa	Archer et al., 2017
			1013.2		River downstream	South Africa	Archer et al., 2017
		54	140	1200	Raw influent	USA	Blair et al., 2013b
		17	180	810	Final effluent	USA	Blair et al., 2013b
			250	3000	Influent	Australia	Watkinson et al., 2009
			50	200	Effluent	Australia	Watkinson et al., 2009
			8	2000	Surface water	Australia	Watkinson et al., 2009
			n.d.	n.d.	Drinking water	Australia	Watkinson et al., 2009
Tetracycline	antibiotic	<BLD		85	Influent	Luxembourg	Pailler et al., 2009
		<BLD		24	Effluent	Luxembourg	Pailler et al., 2009
		<BLD		8	River	Luxembourg	Pailler et al., 2009
		<BLD		7	River	Luxembourg	Pailler et al., 2009
			n.d.	100	Influent	Australia	Watkinson et al., 2009
			n.d.	20	Effluent	Australia	Watkinson et al., 2009
			n.d.	80	Surface water	Australia	Watkinson et al., 2009
			n.d.	n.d.	Drinking water	Australia	Watkinson et al., 2009
Trimethoprim	antibiotic	<BLD		<BLD	River	United Kingdom	Kasprzyk-Hodern et al., 2007

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Trimethoprim		1		126	River	United Kingdom	Kasprzyk-Hodern et al., 2008
		n.d.		n.d.	Tap water	Portugal	Paíga et al., 2017
		n.d.		n.d.	Bottled water	Portugal	Paíga et al., 2017
		n.d.		n.d.	River	Portugal	Paíga et al., 2017
		n.d.		n.d.	Influent	Portugal	Paíga et al., 2017
		n.d.		59.3	Effluent	Portugal	Paíga et al., 2017
				80	Influent	Sweden	Bendz et al., 2005
				40	Effluent	Sweden	Bendz et al., 2005
				151	Surface water	Spain	Gracia-Lor et al., 2011
				232	Effluent	Spain	Gracia-Lor et al., 2011
		<10		36	River upstream	United Kingdom	Ashton et al., 2004
		<10		42	River downstream	United Kingdom	Ashton et al., 2004
		<10		1288	Effluent	United Kingdom	Ashton et al., 2004
		101		277	Influent	South Korea	Behera et al., 2011
		13		154	Effluent	South Korea	Behera et al., 2011
			383		River upstream	South Africa	Archer et al., 2017
			898.7		River downstream	South Africa	Archer et al., 2017
		18	49	590	Raw influent	USA	Blair et al., 2013b
		<BLD	120	660	Final effluent	USA	Blair et al., 2013b
			430	4300	Influent	Australia	Watkinson et al., 2009

Compound	Class	Min concn (ng/L)	Med concn (ng/L)	Max concn (ng/L)	Compartment	Country	References
Trimethoprim			10	250	Effluent	Australia	Watkinson et al., 2009
			3	150	Surface water	Australia	Watkinson et al., 2009
			n.d.	n.d.	Drinking water	Australia	Watkinson et al., 2009
Venlafaxine	Antidepressant	n.d.		n.d.	Tap water	Portugal	Paíga et al., 2017
		n.d.		n.d.	Bottled water	Portugal	Paíga et al., 2017
		n.d.		n.d.	River	Portugal	Paíga et al., 2017
		<BLD		15.4	Influent	Portugal	Paíga et al., 2017
		91.9		170.9	Effluent	Portugal	Paíga et al., 2017
		336		415	Influent	USA	Subedi & Kannan, 2015
		339		480	Effluent	USA	Subedi & Kannan, 2015
		526		1115	Raw wastewater	Canada	Metcalfe et al., 2010
				520	Influent	Spain	Gracia-Lor et al., 2010
				300	Effluent	Spain	Gracia-Lor et al., 2010
				575	Surface water	Spain	Gracia-Lor et al., 2011
				875	Effluent	Spain	Gracia-Lor et al., 2011
		99.5		672	River	USA	Schultz et al., 2010
		146		690	River	USA	Schultz et al., 2010
			35.4		River upstream	South Africa	Archer et al., 2017
			94.6		River downstream	South Africa	Archer et al., 2017

1.4 Sources of contamination

Pharmaceuticals are organic chemical compounds that are used both in human and veterinary medicine and they can reach the aquatic environment directly or indirectly in disparate ways (Ayscough et al., 2000; Daughton & Ternes, 1999; Ebele et al., 2017; Fent et al., 2006; Halling-Sørensen et al., 1997; Klatte et al., 2017; Santos et al., 2010; Yang et al., 2017), (Figure 1.2). Pharmaceuticals in the environment may be the result of negligible release resulting from the improper disposal of expired medicines from hospitals and medical centres, or from the manufacturers themselves (Ayscough et al., 2000). Moreover, pharmaceuticals may also be discharged into the environment after being metabolised and excreted by human or animals (Tambosi et al., 2010). Additionally, pharmaceuticals can be directly introduced into aquatic ecosystems by direct administration through aquaculture practices (Boxall, 2018).

In mammals and aquatic vertebrates, after administration, pharmaceuticals compounds may undergo changes in their structure through metabolic reactions mediated by the P450 microsomal oxidase system (Daughton & Ternes, 1999). These reactions are commonly subdivided in reactions of Phase I and reactions of Phase II (Figure 1.3). Phase I reactions are usually either oxidation, reduction or hydrolysis. The resulting metabolite may then undergo Phase II reactions, where it might be conjugated with either glucuronic acid, sulphate or amino acids

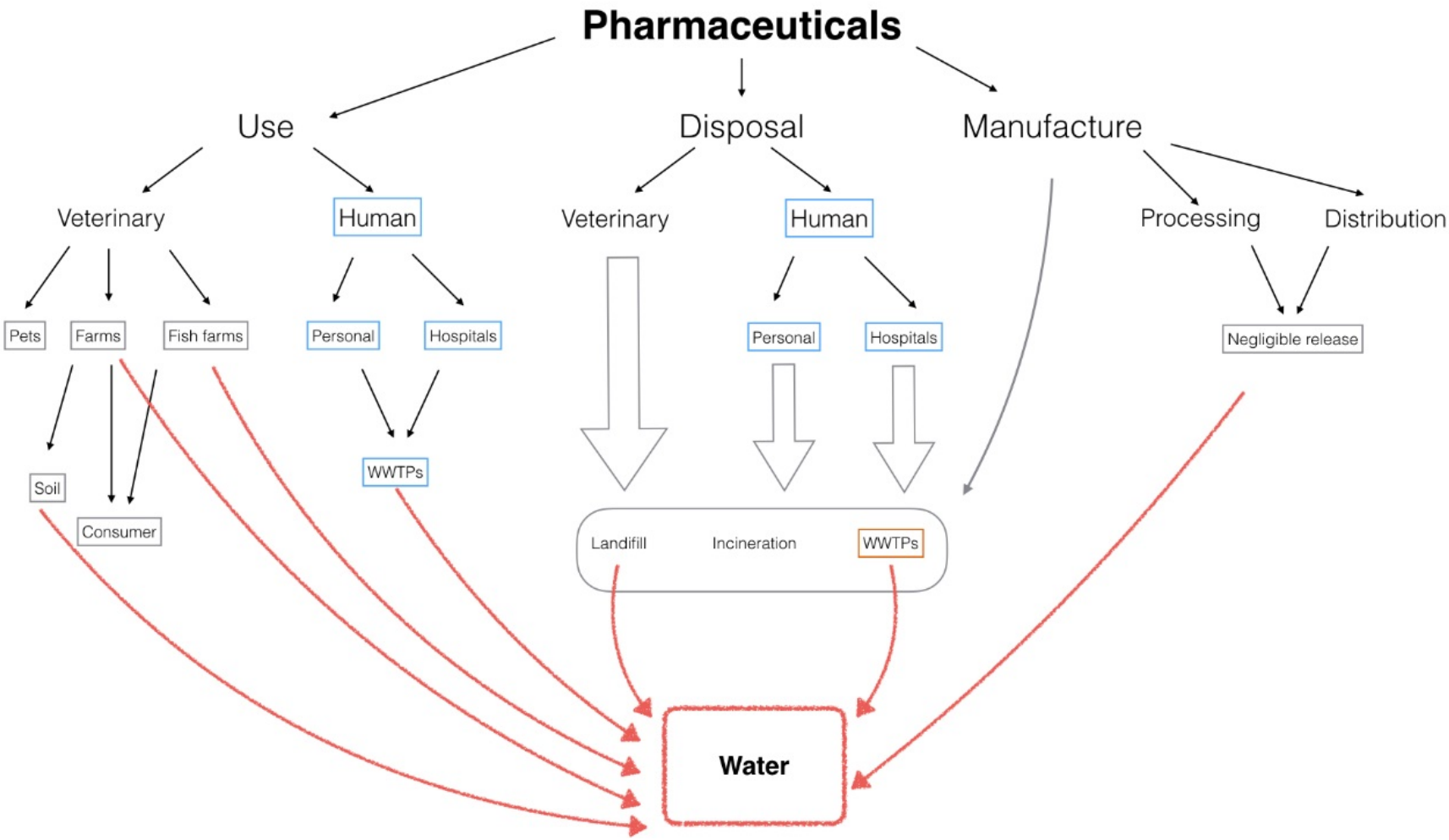

Figure 1.2. Sources of pharmaceuticals into the aquatic environment (Adapted from Ayscough et al., 2000).

Phase II reactions perform the function of increasing the hydrophilic properties in order to facilitate their excretion (Daughton & Ternes, 1999.; Ayscough et al., 2000; Regoli & Giuliani, 2014).

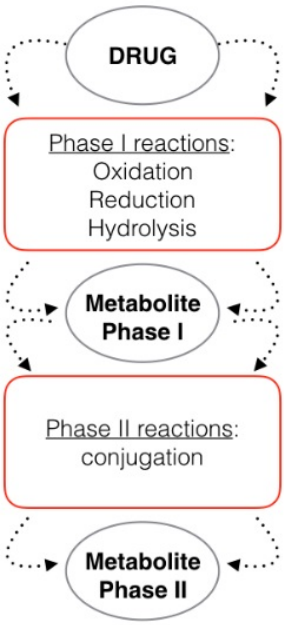

Figure 1.3. Metabolic pathway mediated by the P450 microsomal oxidase system (Adapted from Daughton & Ternes, 1999).

Once pharmaceuticals are metabolised by the organism, they are excreted through urine and feces as a mixture of the original compound and its metabolites (Cunningham et al., 2006; Nebot et al., 2015; Briones et al., 2016). Indeed, most pharmaceutical compounds are only partially metabolised and sometimes not at all (e.g. the antidiabetic metformin), and consequently, they are excreted in their original form (Hirsch et al., 1999; Ayscough et al., 2000). In some other cases, they are excreted as conjugated metabolites and converted back into the original compound after reaching the aquatic environment (Hirsch et al., 1999; Ayscough et al., 2000; Carballa et al., 2004) or through biological process in the wastewater treatment plants (WWTPs), (Blair et al., 2015). The majority of pharmaceuticals are discharged into the sewage system and carried to wastewater treatment facilities (Verlicchi et al., 2012).

Even though aquaculture is a practice that has been carried out for millennia, it is only in the last century that it has rapidly developed, in response to an

exponential growing population and consequent, fish/ shellfish demand (Sapkota et al., 2008). Pharmaceuticals may be added to the food pellet as medicinal products and growth promoters (Boxall, 2018; Daughton & Ternes, 1999; Gaw et al., 2014; Hirsch et al., 1999; Zenker et al., 2014) and this is particularly true in many Asian countries, where most of aquaculture production takes place (Binh et al., 2018). Medications may be used to cure diseases, infections and reduce the risk of possible disease outbreaks, that commonly are a direct consequence of unhealthy breeding conditions (Binh et al., 2018; Gaw et al., 2014; Sapkota et al., 2008). In fact, Gaw et al. (2014) asserted that up to 75% of medicines are likely to be released directly into the environment in their active form. Nevertheless, WWTPs are considered to be the main route and discharge source of pharmaceuticals contamination in freshwater ecosystems (Gogoi et al., 2018; Tambosi et al., 2010).

1.4.1 Wastewater treatment

Wastewater treatment plants (WWTPs) were initially designed to treat and remove biodegradable carbon, phosphorus and nitrogen compounds as well as microorganisms from wastewater (Couto et al., 2019). However, nowadays many different chemicals and contaminants are discharged into sewage system and unfortunately, most wastewater facilities are not designed to handle their disposal (Daughton & Ternes, 1999; Deblonde et al., 2011; Tambosi et al., 2010; Yang et al., 2017), especially because of their low concentrations and the presence of a great variety of different compounds.

Pharmaceuticals are characterised by diverse chemical and physical properties, such as solubility, volatility, absorbability, biodegradability and polarity (Taheran et al., 2018; Verlicchi et al., 2012). WWTPs are supposed to purify the initial raw sewage that is then converted into an effluent that might be either discharged into the environment or reused.

WWTPs may be classified by the influent origin: sewage, leachate, industrial or agricultural wastewater. Sewage treatment plants or WWTPs mainly process household/ municipal wastewater, urban run-off, storm water and liquid wastes from industries, where usually the wastes are pre-treated at the factories. Traditional WWTPs comprise several different processes (i.e. chemical, physical

and biological) that are involved in the purification cycle and are gathered in four stages (Figure 1.4): (1) preliminary treatments, (2) primary treatments, (3) secondary treatments, and occasionally, (4) Tertiary treatments (Peake et al., 2015). The preliminary treatment of the raw sewage is the first step in a WWTP, during which, material that might damage the infrastructure of the primary treatment is removed (e.g. leaves, branches, trash and random objects). Grit removal, flow equalisation, fat and grease removal are usually important processes in this stage.

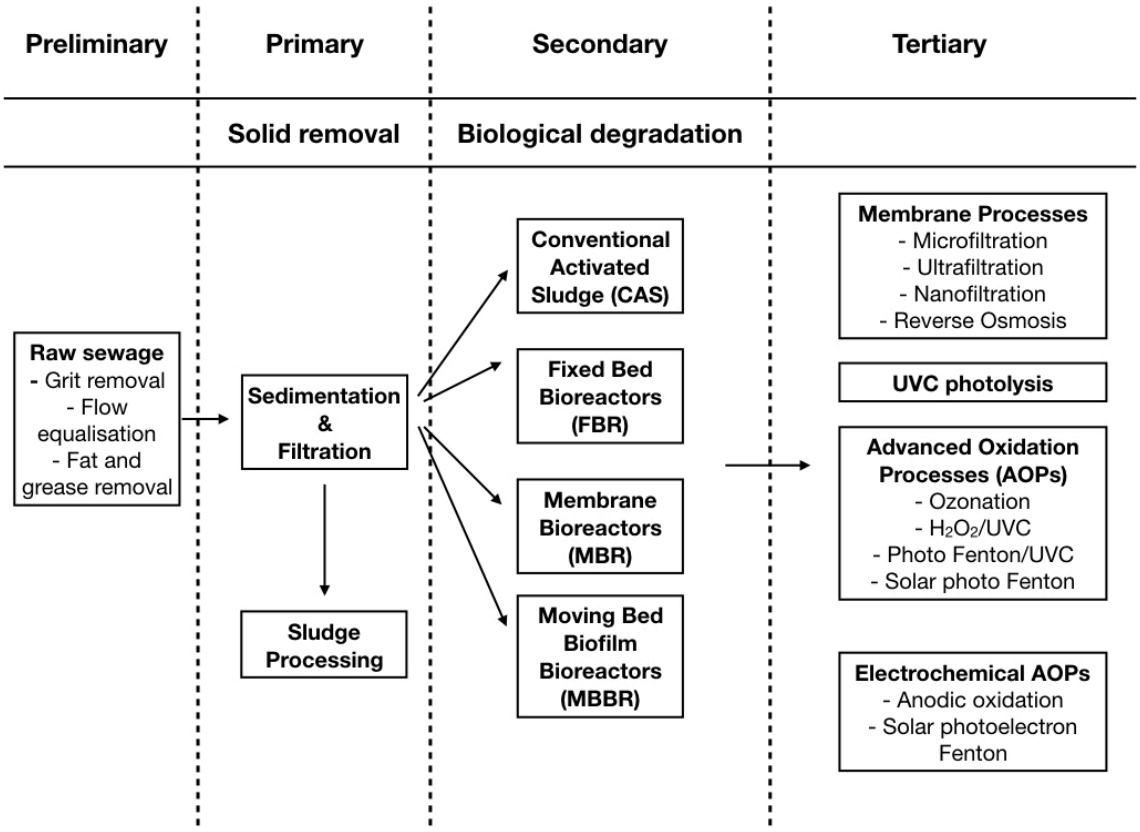

Figure 1.4. Graphical explanation of the different wastewater treatment stages.

During the primary treatment, the solid waste of the influent is separated, commonly by filtration and sedimentation. In this stage, the sludge is collected and it will be treated separately. Sludge may be re-used to fertilse fields, incinerated or landfilled and if pharmaceuticals are not completely removed, they can reach the environment and the aquatic ecosystem through run-off waters (Boxall et al., 2012; Boxall, 2018).

Secondary treatments involve the biological degradation (aerobically or anaerobically) of organic substances and can be achieved by several different techniques including, conventional activated sludge (CAS), oxidation ditches, fixed bed bioreactors (FBR), membrane bioreactors (MBR) and moving bed biofilm reactors (MBBR) (Gogoi et al., 2018; Peake et al., 2015).

Tertiary treatments are sometimes applied in WWTPs and this could be done through several different processes: (1) membrane processes (e.g. microfiltration, ultrafiltration, nanofiltration and reverse osmosis), (2) UVC photolysis, (3) Advanced Oxidation Processes (AOPs), (e.g. ozonation, H2O2/ UVC, photo Fenton/ UVC and solar photo Fenton) (4) Electrochemical AOPs (e.g. anodic oxidation and solar photoelectron Fenton), (Moreira et al., 2016). Tertiary treatments might help to enhance the removal rate of recalcitrant micropollutants, such as pharmaceuticals, however they might sometimes increase the risk of more toxic and dangerous compounds forming (e.g. ozonation), (Boxall et al., 2012; Schlüter-Vorberg et al., 2015). Nowadays, tertiary treatments are usually required for those areas that are designated as Sensitive Areas (Defra, 2012).

In WWTPs, pharmaceuticals and their metabolites behave differently, depending on their physicochemical properties (Evgenidou et al.,2015). They may undergo partial or complete mineralisation and can be transformed into water and carbon dioxide (Richardson & Bowron, 1985). Lipophilic compounds may bind to solid matter (e.g. sludge), (Halling-Sørensen et al., 1998), whereas hydrophilic and polar substance may remain dissolved in the water and are not removed by WWTPs (Carballa et al., 2004).

The removal efficiency of pharmaceuticals in WWTPs not only depends on the chemical properties of the pollutant and the type of treatment applied, but is also related to seasonal conditions (Carballa et al., 2004; Evgenidou et al., 2015). Their efficiency tend to be lower during winter months (Verlicchi et al., 2012; Vieno et al., 2005) because of heavy rainfall and lower water temperatures, both of which influence the biodegradation rate. Additionally, the removal efficiency of

some pharmaceuticals decreases at the beginning of autumn, when the temperature drops and health problems increase, enhancing pharmaceuticals consumption (Ziylan & Ince, 2011; Petrie et al., 2015).

1.5 Current legislation and Environmental Risk Assessment (ERA)

On the 26th January 1965, in reaction to the Thalidomide tragedy in the early 1960's, the European Economic Commission (European Commission since 1992) issued the first European pharmaceuticals directive: Council Directive 65/65/EEC1. This Directive stated that every Member State's authority has to issue an authorisation for any medicinal product that goes on the market, which aimed to protect and ensure public health. Since then, the Directive 65/65/EEC1 was modified several times to achieve a single EU-wide market for human pharmaceuticals.

In January 1995, a new European system for authorising medical products came into effect through Directive 93/41/EEC. It offered two routes of authorising medicinal products: through the European Agency for Evaluation of Medicinal Products (EMEA) or through a "mutual recognition" procedure in the Member State. The EMEA, known nowadays as European Medicines Agency (EMA) was established in 1995 and its main task is to co-ordinate the scientific evaluation for the safety, effectiveness and quality of medicinal products. In accordance with Article 8(3) of Directive 2001/83/EC an Environmental Risk Assessment (ERA) should accompany the application for marketing any new medicinal product, in particular, if the product contains vitamins, electrolytes, amino acids, peptides, proteins, carbohydrates and lipids as pharmaceuticals ingredient(s).

An ERA is a procedure that aims to evaluate the potential risk that a substance or product poses to the environment and it is a phased procedure, consisting of two phases. In Phase I, the Predicted Environmental Concentration (PEC) is calculated. If the PEC value is below 0.01 µg/L, the medicine is not considered a hazardous substance. Nowadays, the 0.01 µg/L action limit is mainly based on acute toxicity experiments, performed using organisms that belong to different trophic levels such as algae, zooplankton, other invertebrates and fish. But if the

PEC value is equal or above 0.01 μg/L, then Phase II analyses should be carried out. In this step, the PEC/ PNEC (Predicted no-effect concentration) ratio is calculated, using the base set of data and the predicted environmental concentration. If the ratio PEC/ PNEC for the medicine is less than 1, then further testing on the aquatic system is not required, and the drug may be considered harmless. In contrast, if the ratio is greater than 1, further evaluation is necessary in order to ensure that the compound is safe to use (EMEA, 2006).

Even though the PEC/ PNEC ratio is a valid evaluation method, the considered abiotic variables used to calculate PEC can vary markedly throughout the year, depending on the season, the number of inhabitants of the selected area and on the pharmaceutical class. In addition, the PEC/ PNEC ratio does not cater for the various treatment methods used by each WWTP. Also, the PEC and PNEC values are commonly estimated through acute toxicity tests, which are easily computable and replicable, however they may not reflect the true environmental conditions. For example, Minguez et al. (2014) investigated the acute toxicity of several antidepressants and they reported EC_{50} values ranging between 1.15 and 141.8 mg/L. These concentrations are more than one order of magnitude higher than the actual reported environmental concentrations (Table 1.1). Moreover, there are several studies showing how far lower concentrations of antidepressants can induce alterations (e.g. behavioural alterations) in non-target aquatic organisms (Bidel et al., 2016; Fong & Ford, 2014; Fong & Molnar, 2013). Moreover, the potential bioaccumulation and biomagnification of pharmaceuticals in aquatic organisms should be studied and integrated into ERAs, in order to have a broader understanding of the prospective environmental toxicity of these compounds (Brausch & Rand, 2011).

On 23rd October 2000, the European Commission issued a new Council Directive (Council Directive 2000/60/EC), in order to achieve Good Environmental Status for all European Union waters by 2015. The Directive aimed to establish a common framework for water protection and management. In 2001 the Directive 2000/60/EC was modified with the amending Decision

2455/2001/EC, that established a list of priority substances, replacing the list published in the Commission Communication of 22nd June 1982.

In 2008 a new amending Directive (2008/105/EC) was issued, establishing environmental quality standards, and in 2013 the European Union Water Framework Directive 2013/39/EU was issued, amending Directives 2000/60/EC and 2008/105/EC, proposing a reviewed list of 45 priority substances with environmental quality standards (EQS) to be respected in aquatic ecosystems. In the watch-list of priority substances, three pharmaceutical compounds were included for the first time: the anti-inflammatory Diclofenac, the synthetic hormones 17α-ethinyl estradiol and 17ß-estradiol (European Commission, 2013).

In March 2015, Decision 2015/495/EU was published, establishing a watch list of substances for Union-wide monitoring, which was then implemented by the Decision 2018/840/EU. At the same time, the Global Water Research Coalition published a list of different pharmaceuticals (e.g. carbamazepine, naproxen, sulfamethoxazole, ibuprofen, diclofenac, gemfibrozil and erythromycin) considered to be of concern for water environments (Global Water Research Coalition, 2008).

Monitoring programmes have been established in several countries. One such programme is the Chemical Investigation Program (CIP) in the UK where the water industry monitors concentrations of priority substances and pharmaceuticals included in wastewater influents and effluents, as well as assessing the potential impact that these substances can have on natural receiving waters (Gardner et al., 2013), in order to meet the UK obligations under the Water Framework Directive (WFD).

1.6 Pharmaceuticals in the freshwater environment

Freshwater ecosystems have played a central and prominent role in human civilisation, providing freshwater not only for drinking purposes, but also for irrigation, transportation, power production and waste disposal (Strayer & Dudgeon, 2010). Rivers are also a source of food (e.g. fish) and river surroundings are often suitable sites for homes and industries (Strayer &

Dudgeon, 2010). Even though rivers account for only 0.8% of the Earth surface area, rivers are home to 9.5 % (~125.000) of all known animal species (Dudgeon et al., 2006), making them hotspots of biodiversity (Strayer & Dudgeon, 2010). Unfortunately, centuries of human exploitation of freshwater ecosystems have had detrimental consequences. WWTPs' effluents are considered to be the main route of contamination, especially for riverine environments (Gogoi et al., 2018).

Improvements in analytical techniques have helped ecotoxicologists detect pharmaceuticals in the environment (Table 1.1), and investigate their impact, leading to increased numbers of publications and studies (Figure 1.1) (Hughes et al., 2012). However, the majority of these studies are based on acute tests (<96 h), using lethal endpoints (e.g. median lethal dose, LC_{50}) at high concentrations, (Santos et al., 2010). Most pharmaceuticals are designed to cause minimal acute toxicity, therefore the majority of these compounds are lethal in short-term exposures only at high doses. It is only recently that researchers have focused on understanding the possible impact of prolonged exposure at environmentally realistic concentrations, which usually fall into the ng/l to µg/L range. Nowadays, relevant concentrations of pharmaceuticals have been tested on a wide variety of non-target aquatic organisms, such as fish (e.g. Gaworecki & Klaine, 2008; McCallum et al., 2019), algae (e.g. Geiger et al., 2016), gastropods (e.g. Boisseaux et al., 2017; Contardo-Jara et al., 2011; Jacob et al., 2019) and amphipods (e.g. Gómez-Canela et al., 2016). In fact, aquatic ecotoxicologists may adopt different model species to investigate the toxicity of different compounds. Bivalves are a prominent example of aquatic model organisms (e.g. *Mytilus edulis* and *Mytilus galloprovincialis*) that are considered extremely valuable indicators of pollution and their responses to a wide range of contaminants have been extensively studied (Canesi et al., 2012). Similarly, aquatic Gammarids have been used for decades in ecotoxicology to understand the effects of inorganic and organic substances (Kunz et al., 2010).

1.7 Freshwater Gammarids as test species

Even though most Amphipoda are from marine environments, there ar 2e more than 1,500 freshwater amphipod species, that account for almost 20% of their total diversity (Väinölä et al., 2008). The sub-order Gammaridea represents

the most diverse group with more than 4,500 species ranging from marine, to freshwater and terrestrial habitats (Kunz et al., 2010). Among the sub-order Gammaridea, the genus *Gammarus* comprises more than 100 freshwater species that are mainly distributed in freshwater streams in the Northern Hemisphere (Karaman & Pinkster, 1977a), and in particular the two species *Gammarus pulex* and *Gammarus fossarum* are widely distributed around Europe and Northern Asia (Karaman & Pinkster, 1977a). Organisms of the genus *Gammarus* spp. usually find shelter under rocks, within gravel and among living and dead vegetation (Fitter & Manuel, 1994), that not only can serve as a shelter from predators but can also function as a food source (MacNeil et al., 1997). In the ecotoxicological community, freshwater Gammarids are well recognised as good representative test species as they present all the main characteristics of ecotoxicological model organisms: (1) wide distribution; (2) sensitive; (3) representative; (4) ecological key role; and (5) subjected to direct exposure to contaminants. For all these reasons, *G. pulex* was chosen as model organisms for this PhD project. Moreover, *G. pulex* was easily collectable from freshwater streams in the local area of the university.

Gammarids have also been proven to be fairly sensitive to pollutants, both organic and inorganic (Schmidlin et al., 2015b; Vellinger et al., 2012; Wogram & Liess, 2001). Moreover, Gammarids are also noteworthy prey for fish, birds and amphibians (MacNeil et al., 1999a) and they play a key role in the decomposition of coarse particulate organic matter (CPOM) into fine particulate organic matter (FPOM) (Cummins & Klug, 1979). Consequently, they link different trophic levels of the aquatic food web, making them keystone species (Sutcliffe, 1983a; Woodward et al., 2008) and shifts in their population may have profound implications for the entire ecosystem (Hodkinson & Jackson, 2005). Over the years, Gammarids have been used as ecotoxicological models, and also as sentinel species to assess water quality (Garcia-Galan et al., 2017).

Freshwater *Gammarus* spp. are characterised by a short life-cycle (usually around 1-2 years). *G. pulex* is characterised by an evident sexual dimorphism, which allowed us to perform certain experiment exclusively on males.

Male specimens can grow up to 2.0 cm in length, whereas females are usually shorter (Lahive et al., 2015; Kunz et al., 2010). They are laterally compressed and they usually present a semi-circular shape when resting (Glazier, 2009).

The body can be divided into three main parts: (1) head, (2) pereon, and (3) pleon (Figure 1.5).

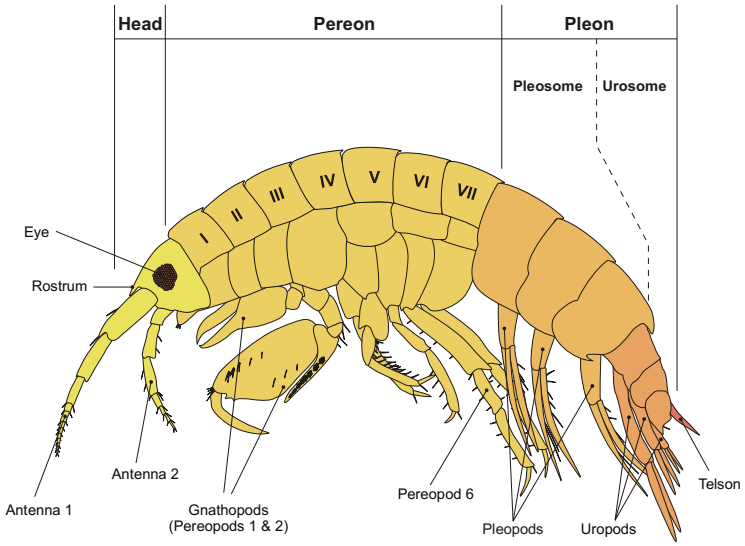

Figure 1.5. Anatomy of a male amphipod (Lycaon, 2006).

The head has two pairs of antennae, with the second pair being shorter than the first, a mouth, and compound eyes. The pereon is equipped with 7 pairs of legs known as pereopods and they are used for swimming and grasping. In adult males the first two pairs of pereopods are enlarged and they are called gnathopods. Gnathopods can be used for feeding, grooming and grasping females (MacNeil et al., 1997). The pleon can be subdivided into the pleosome and the urosome. The pleosome includes three pairs of appendices called pleopods whereas the urosome has three pairs of appendices called uropods. Posterior to the pleon there is the telson. Both pleopods and uropods are used in locomotion as well as for circulating water (Glazier, 2009).

Gonads are situated in the pereon, from segment 2 to 7, and they are paired tubular organs (Sutcliffe, 1993a). Ovary size changes with state of maturation and oviducts arise at pereon segment 5, whereas each male testis expands to form a canal at pereon segment 6.

Females are available for mating only for a short time, immediately after moulting, whereas *Gammarus* males are available for most of their moult cycle (Sutcliffe, 1993b). Males will start precopulatory mate guarding once they encounter a female about to moult (Ridley, 1983). During precopulatory mate guarding, the male usually holds, by using the first pair of gnathopods (Figure 1.5 and Figure 1.6), and carries a female under his ventral surface (Borowski, 1984) (Figure 1.7). This process may last for several weeks until the female moults and they can

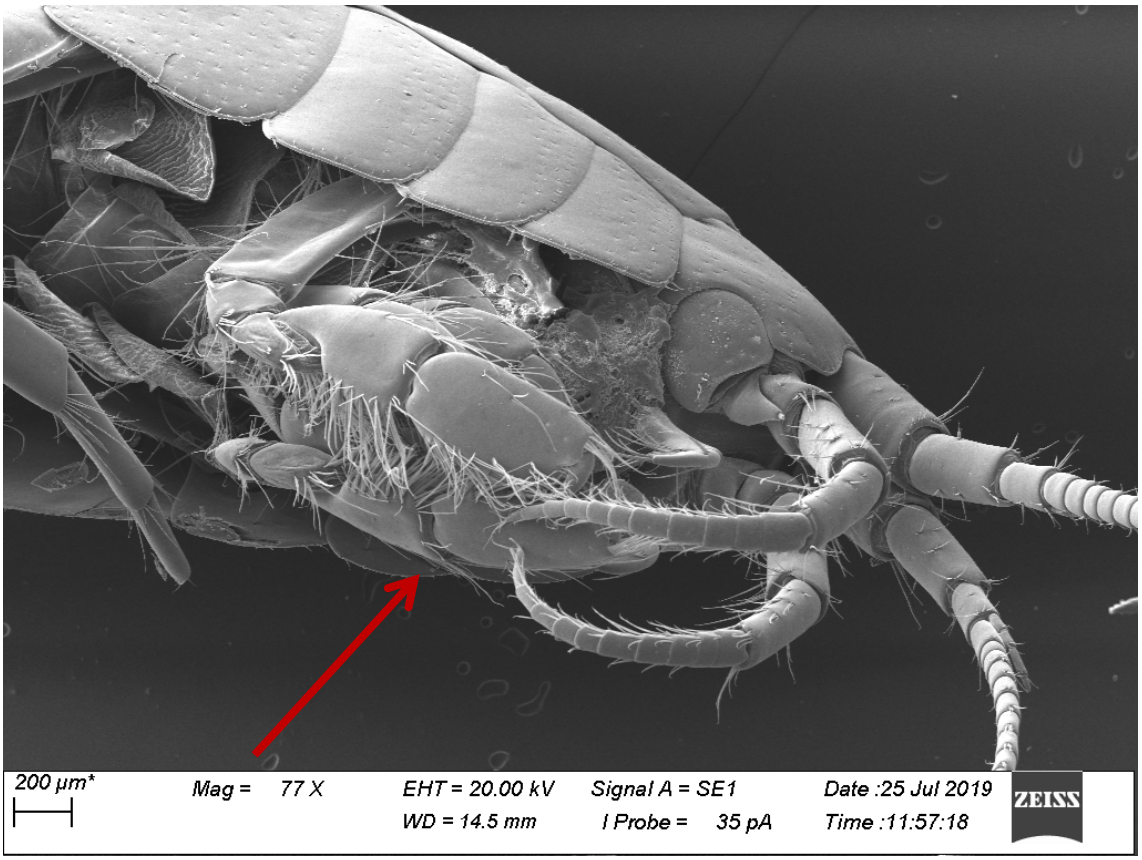

Figure 1.6. Scanning electron microscopy photo of a male specimens of *Gammarus pulex*. The red arrow is to indicate the organism's gnathopods.

mate. Thereafter, they separate and the female carries the growing offspring in a brood pouch for over a month until they are released (Kunz et al. 2010).

Figure 1.7. *Gammarus* sp. precopula pair (Glazier, 2009).

1.8 *Gammarus* spp. in ecotoxicological studies

Gammarus spp. are sensitive keystone species that have a prominent role in lotic ecosystems and are frequently used in ecotoxicological studies, to assess the acute and sublethal toxicity. The species have been used to test the effects of many different substances, such as pesticides, heavy metals, herbicides and pharmaceuticals.

Blockwell et al. (1996) investigated the effect of sublethal concentrations of the organochlorine insecticide, lindane, on the growth of *G. pulex* juveniles over a period of 14 days. Growth was significantly inhibited in organisms exposed to the highest nominal concentration of 6 µg/L. Adam et al. (2009) investigated the lethality of the two fungicide propiconazole and tebuconazole, the wood preservative 3-iodo-2-propynyl butyl carbamate (IPBC) and the pyrethroid insecticide cypermethrin. The compounds were tested individually and in mixtures. LC_{50} was measured after 24 h, 48 h, 72 h and 96 h and it was shown

that the mixtures had a greater toxicological effect compared to the individual compounds.

In another investigation, adults and juvenile *G. fossarum* and *G. pulex* were used to investigate the acute toxicity of another pyrethroid insecticide (deltamethrin) (Adam et al., 2010). Adult specimens of *G. fossarum* were found to be twice as sensitive compared to adult *G. pulex*, and juveniles of both species were up to twenty-two times more sensitive to deltamethrin than adults. The neonicotinoid insecticide, imidacloprid, has been shown to inhibit the feeding rate of *G. pulex* at concentrations ≥ 30 µg/L (Agatz et al., 2014). Specimens of *G. fossarum* have also been used for *in situ* investigations to assess the level of Cd, Hg, Pb and Ni contamination in 117 sites around France (Ciliberti et al., 2017).

G. pulex specimens were used to investigate the LC_{50} of Cd over a period of 11 days (Felten et al., 2008a). Organisms exposed to Cd had a whole body concentration of Cd that was significantly higher compared to the controls. Moreover, *G. pulex* was also exposed to relevant concentrations of Cd (0 µg/L, 7.5 µg/L and 15 µg/L) and sublethal endpoints included locomotor activity and feeding rate. These are just a few examples of the investigations that have been undertaken using *Gammarus* spp. as test organism in the last few decades. *Gammarus* spp. specimens used for scientific experiments may be cultured in the laboratory through breeding programmes (e.g. Blockwell et al., 1996; Blockwell et al., 1998; Bloor et al., 2005; McCahon & Pascoe, 1998a,b,c), or more commonly, organisms are collected in the wild and left to acclimate to laboratory conditions for a certain period of time. The use of laboratory bred animals might be considered more appropriate as their past life history is known and they have not come into contact with pollution, in contrast to wild organisms (Liber et al., 2007).

However, due to their pollution-free life history, laboratory bred animals might be more or less sensitive to contaminants. Consequently, their responses may not reflect real environmental conditions (Gerhardt et al., 2004), which could result in either an underestimation or overestimation of a compound's toxicity. In addition,

laboratory breeding programmes may lead to a reduced genetic variability (Liber et al., 2007).

1.8.1 *Gammarus* spp. and pharmaceuticals

Over the years, *Gammarus* spp. have been adopted as experimental test species to study the effects of many different pharmaceuticals. A recent investigation examined how mixtures of common surfactants can influence the bioconcentration of two different pharmaceuticals compounds (sulfamethoxazole and oxazepam) in *G. fossarum* (Garcia-Galan et al., 2017). Similarly, Miller et al. (2017) studied the uptake and elimination kinetics of *G. pulex* of different drugs (sulfamethazine, carbamazepine, diazepam, temazepam, trimethoprim, warfarin, metoprolol, nifedipine and propranolol), while also investigating the bioconcentration factors (BFs) after 96 h exposure and the depuration phase.

Pharmaceuticals uptake from water was also studied and demonstrated by Meredith-Williams et al. (2012) using three different freshwater species (*G. pulex*, *Notonecta glauca* and *Planorbarius corneus*). In their study, each pharmaceutical (5-fluorouracil, carvedilol, diazepam, moclobemide, carbamazepine and fluoxetine) was labelled either with 14C or 3H in order to assess the uptake, the depuration rate and bioconcentration factors. De Lange et al. (2006) used *G. pulex* as an ecotoxicological model to study how their activity rate changed in response to a short-term exposure to different pharmaceuticals, including the antidepressant, fluoxetine, the anti-inflammatory drug, Ibuprofen and the anti-epileptic drug, carbamazepine. Their study was carried out using the Multispecies Freshwater Biomonitor (MFB), a technique based on impedance conversion that can be used to quantitatively monitor different behaviours simultaneously. The results showed a significant drop in activity in those organisms exposed to low concentrations of fluoxetine and ibuprofen (10-100 ng/L), whereas exposure to higher concentrations did not reveal any meaningful changes. Exposure to carbamazepine presented a similar trend in activity, but was not significant compared to the control (De Lange et al., 2006).

1.8.2 Sublethal endpoints with a focus on behaviour

Acute lethality tests with *Gammarus* spp. are now considered to be inadequate and non-representative of the real environmental conditions. Therefore, the use of sublethal endpoints (e.g. changes in behaviour), that can provide a more relevant understanding of the impact of different pollutants at environmentally realistic concentrations, has increased (Coulaud et al., 2011).

Behavioural endpoints have been used in aquatic ecotoxicology for several decades (Bae & Park, 2014), as they can provide a link between changes in environmental conditions and physicochemical alteration in the organism (Gerhardt, 2007). They can also be an indicator of the fitness of the organism, and more broadly, of the entire population. Indeed, behaviour is the response of the organism to a stimulus (Gerhardt, 2007), which in ecotoxicology could be the presence of a certain contaminant. Improvements in technology and the development of new video tracking devices have allowed aquatic ecotoxicologists to improve and employ behavioral analyses (Bae & Park, 2014) as they are frequently criticised for their lack of standardisation and replicability (Consolandi et al., 2019; Parker, 2016). One such area is feeding behaviour studies, where scientists have employed many different approaches.

Feeding behaviour was chosen as the main behavioural endpoint in this PhD project because it has been broadly adopted in Gammarids ecotoxicity experiments. Freshwater Gammarids play a key role in the decomposition and breakdown of organic matter in lotic environments. Therefore, it is unsurprising that their feeding behaviour is often used as a sublethal endpoint to investigate the impact of different types of pollutants (e.g. Alonso et al., 2009; Bundschuh et al., 2011a; Weber et al., 2018). Feeding assays have been shown to represent real leaf decomposition in the environment (Maltby et al., 2002) and they have been carried out both as *in situ* (i.e. directly in the environment) and *ex situ* (i.e. in the laboratory under controlled conditions) experiments (Bundschuh et al., 2011; Dedourge-Geffard et al., 2009; Maltby et al., 2002; Zubrod et al., 2015). Moreover, feeding behaviour has been chosen as the main sublethal endpoint for this research as it is very cost-effective. In fact, *G. pulex* can be fed with pre-dried laboratory conditioned Alder leaves (*Alnus glutinosa*) and differences in feeding can be estimated after only a 4ew hours (e.g. Hahn & Schulz, 2007). Additionally,

feeding behaviour experiments generally do not require the use of expensive and specific machinery. For this reason, gammarids feeding behaviour has been studied for several decades, whereas other behavioural endpoints (e.g. swimming velocity) have been increasingly adopted in recent years with the development of highly specific and sensitive technologies (Bossus et al., 2014; De Castro-Català et al., 2017; De Lange et al., 2009; Kohler et al., 2018).

However, even though *Gammarus* spp. feeding behaviour has been used for several decades (e.g. Felten et al., 2008a; Ganser et al., 2019; Hargeby & Petersen, 1988), there is not yet an universal standardised methodology. Differences in the protocols can be found in the acclimation conditions, the food preparation and in the quantification method, where different equations are used (Consolandi et al., 2019).

Behavioural analyses are often characterised by higher inner variability, making replicability of the experiment more difficult. Therefore, biomarkers are frequently performed alongside, not only to strengthen the results, but also to better understand the relationship between a particular environmental stressor (e.g. a specific pharmaceutical), behavioral alteration and the physiological response of the organism.

1.9 Aims of this thesis

The aim of this PhD project was to understand the prospective impact that different classes of pharmaceuticals might have on the behaviour of the freshwater amphipod *G. pulex*. This was achieved by studying the variation in sublethal behavioural endpoints and how these different methodologies compare, by focusing specifically on feeding behaviour.

Four specific objectives were addressed:

1. To review the literature regarding *Gammarus* spp. feeding behaviour as a sublethal endpoint in ecotoxicology, by highlighting the disparities in the current published methodologies, and to help develop a standardised protocol (Chapter 2).
2. To examine the implications of using different feeding equations to estimate the feeding rate of *G. pulex* when exposed to the antidiabetic

drug metformin, and to understand if different published equations generate equivalent results. Moreover, to investigate if metformin affects the feeding behaviour and swimming velocity of *G. pulex* (Chapter 3).

3. To understand if a mixture of two different antibiotics, sulfamethoxazole and trimethoprim, might have an impact on the natural bacterial communities in the water and disrupt the natural leaf conditioning process, and consequently alter the feeding rate of *G. pulex* (Chapter 4).
4. To address the impact of the antidepressant venlafaxine on different behavioural patterns (feeding rate, movement, ventilation and swimming velocity)3 of *G. pulex*, by using different behavioural tracking devices (The Multipecies Freshwater Biomonitor and the DanioVision™ observation chamber) and to evaluate the comparability between protocols (Chapter 5).

The findings from each piece of research were then analysed and compared (Chapter 6) to reach a conclusion on the ecological impact of different classes of pharmaceuticals on *G. pulex*, and to determine the effective validity of behavioural endpoints, specifically feeding behaviour.

Chapter 2: Feeding behavioural studies with freshwater *Gammarus* spp.: the importance of a standardised methodology[3]

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Author contribution:

All the data presented in this chapter were reviewed and interpreted by Consolandi, G. All authors contributed and revised the final manuscript before submission. The review was accepted and published in *Reviews of Environmental Contamination and Toxicology*

2.1 Abstract

Feeding behaviour of freshwater Gammarids has been used for several decades as a sublethal toxicity endpoint. Feeding behaviour has been demonstrated to be an effective endpoint, but there is not a standardised assay. This paper aims to review the existing published literature to highlight the methodological discrepancies in feeding behavioural studies (both *in situ* and *ex situ*). Key discrepancies in the acclimation period were temperature, duration, media, light/dark cycles and the characteristics of the test organisms. Interestingly, the food preparation method and the choice of feeding rate equation were also diverse. Non-standardisation of any of these factors could influence the outcome of the experiment and render a comparison between studies difficult. There is an undeniable need for scientific discussion and agreement on a standardised protocol for feeding behavioural studies, to ensure that all future studies are directly comparable and to enhance the usefulness of feeding assays as a biomonitoring tool to assess water quality.

Keywords: Acclimation, *Alnus glutinosa*, Amphipods, Behaviour, Conditioning, Crustacea, Ecotoxicology, *ex situ*, Feeding assays, Feeding rate, Feeding rate equations, Gammarids, *Gammarus*, *Gammarus fossarum*, *Gammarus pulex*, *in*

[3] The published paper can be found in Appendix A

situ, Invertebrates, Leaves, Methodology, Standardised methods, Standardised protocol, Sublethal endpoint, Toxicity testing, Water quality, Water quality monitoring

2.2 Introduction

Freshwater Gammarids are common leaf-shredding detritivores, and they usually feed on naturally conditioned organic material, in other words leaf litter that is characterised by an increased palatability, due to the action and presence of microorganisms (Chaumot et al., 2015; Cummins, 1974: Maltby et al., 2002). *Gammarus* spp. are biologically omnivorous organisms, so they are involved in shredding leaf litter and are also prone to cannibalism, predation behaviour (Kelly et al., 2002) and coprophagy when juveniles (McCahon & Pascoe, 1988). *Gammarus* spp. are keystone species (Woodward et al., 2008), and they play an important role in the decomposition of organic matter (Alonso et al., 2009; Bundschuh et al., 2013) and are also a noteworthy prey for fish and birds (Andrén & Eriksson Wiklund, 2013; Blarer & Burkhardt-Holm, 2016). Gammarids are considered to be fairly sensitive to different contaminants (Ashauer et al., 2010; Bloor et al., 2005; Felten et al., 2008a; Lahive et al., 2015; Kunz et al., 2010); in fact, Amphipods have been reported to be one of the most sensitive orders to metals and organic compounds (Wogram & Liess, 2001), which makes them representative test organisms for ecotoxicological studies and valid sentinel species for assessing water quality status (Garcia-Galan et al., 2017).

Since Gammarids play an important role in the breakdown of organic matter in freshwater environments, it is understandable that their feeding behaviour is often used as a sublethal endpoint, to investigate water quality status and the effects of different contaminant types (Crane & Maltby, 1991). Gammarid feeding activity could be altered by the presence of contaminants in the water, which could potentially alter their food source, influence the organism's biological function and cause abnormal behavioural responses. These types of feeding investigation have been carried out as *in situ* (i.e. directly in the environment) and *ex situ* (i.e. in the laboratory) studies (Bundschuh et al., 2011b; Dedourge-Geffard et al., 2009; Maltby et al., 2002; Zubrod et al., 2015). It has been demonstrated that feeding assays using Gammarids are representative of natural leaf

decomposition in the environment (Maltby et al., 2002) and could be used to assess the effects of chemical contaminants and also understand the consequences of new-generation contaminants, such as plastic debris in freshwater environments (Blarer & Burkhardt-Holm, 2016; Weber et al., 2018). Even though feeding behaviour studies have been carried out for almost half a century, there is a lack of standardisation for both *ex situ* and *in situ* methods. Without standardisation, there is a risk that the effects of a test substance could be under- or overestimated during *in situ* and *ex situ* approaches, which could reduce their usefulness in environmental biomonitoring programs. This paper aims to review the literature on feeding as an endpoint for amphipod ecotoxicology, by highlighting disparities in the published methodologies, and to help develop standardised protocols. Peer-reviewed literature was accessed through search engines, databases and library archives. In general, most feeding studies have reported four main stages: (1) acclimation period, (2) food preparation, (3) exposure and (4) end of the experiment and feeding rate calculation. The aforementioned four stages have been reviewed separately, and the variability of the published methodologies has been considered, in order to draw attention to the current discrepancies in the literature.

2.3 Acclimation conditions

The first stage of an experiment (both *in situ* and *ex situ*) is the acclimation period that should be used to acclimate the organisms to the experimental conditions. However, the acclimation conditions are not always fully disclosed, and when they are, they sometimes contradict the experimental conditions. The reproducibility of an experiment is also highly dependent on many abiotic and biotic factors, which are rarely taken into consideration for Gammarid feeding studies (Coulaud et al., 2011). In the following sections, different variables (duration, temperature, light:dark cycles, type of water and organisms) that could impact the outcome of an experiment have been reviewed separately and summarised in Table 2.1, in order to emphasise the full range of variability within the literature. In some studies, Gammarids are sourced from laboratory breeding programs (e.g. Blockwell et al., 1996; Bloor & Banks, 2006a, b; McCahon &

Pascoe, 1988) and in other studies organisms are collected from the environment (e.g. Bundschuh et al., 2009)

2.3.1 Duration

Acclimation periods vary depending on the study (Table 2.1), for example, Agatz et al. (2014) kept specimens of *Gammarus pulex* in the laboratory for 3 days prior to the start of the experiment, whereas another study left *Gammarus fossarum* organisms to acclimate for 21 days (Garcia-Galan et al., 2017). Typically, the acclimation period used for Gammarids appears to be between 5 and 7 days, but some studies have selected longer intervals up to 35 days (Table 2.1). Agatz and Brown (2014) stated that a 1-day acclimation period helped to reduce the variability of their results by just 1.6%, suggesting that a longer acclimation period could potentially have an even greater impact on reducing the intraspecific variability and consequently strengthen the statistics. Although experimental controls are incorporated into the majority of experimental designs, it becomes difficult to compare published peer-reviewed research when the test organisms have experienced anything between 3 and 35 days of acclimation to laboratory conditions (Agatz et al., 2014; Garcia-Galan et al., 2017), (Table 2.1), even more so when the organisms are used as water quality biomonitors for *in situ* experiments (Table 2.1).

2.3.2 Temperature

During the acclimation period, organisms need to be kept at a constant temperature and with a precise light:dark cycle. Gammarids from temperate countries are usually maintained at a temperature between 10 and 22˚C (Table 2.1). The temperature adopted in an experimental design is often selected to reproduce seasonal conditions, but unfortunately the literature does not always specify the selection criteria. Temperature can have a significant impact on Gammarids and on amphipods in general (Labaude et al., 2017). Foucreau et al. (2014) discovered that temperatures higher than 15˚C altered various physiological parameters in *Gammarus pulex* populations in North France. Southern specimens consumed more oxygen at higher temperatures and had a higher glycogen content, which means they have a higher energy supply. Cold-acclimated organisms consumed more energy and oxygen when they are

exposed to higher temperatures, and they presented a lower heat tolerance (Semsar-kazerouni & Verberk, 2018). Interestingly, Alonso et al. (2009) acclimated their organisms at 15˚C for 4 days, after which time the organisms were transferred to a 20˚C room to acclimate for a further 4 days. Moving organisms from a low to a high temperature could have potentially affected the experimental results (Alonso et al., 2009). Furthermore, temperature plays an important role in the immune system of crustaceans (Le Moullac & Haffner, 2000). Therefore, it is difficult to compare studies where the test animals have been acclimated at different temperatures, as this could have influenced their energy stores or their immune systems, for example. These differences could also be reflected in the organisms' behavioural reactions, which could be incorrectly interpreted as a result of exposure to specific contaminants. In fact, both Nilsson (1974) and Coulaud et al. (2011) reported an increased feeding rate with an increased temperature. The extent of the feeding rate increase was also dependent on leaf species (i.e. *Alnus glutinosa* or *Fagus sylvatica* (Nilsson, 1974). Acclimation temperature plays an even greater role in *in situ* experiments where the chosen temperature should be as close as possible to real-life environmental conditions. Interestingly, Coulaud et al. (2011) linked temperature and feeding rate through a linear regression, in order to better understand the impact of temperature on the Gammarids feeding. It was found that a small increase in mean temperature (from 12 to 13˚C) could enhance the feeding rate by 7.3%.

2.3.3 Light and Dark cycles

The same principle could be applied to the different light:dark cycles used during the acclimation period. The most commonly adopted light:dark cycle is 12:12 h (Table 2.1) that reflects typical equinox conditions. However, some studies acclimate their organisms in total darkness, and in other studies, the adopted cycle is not specified (Table 2.1). Sometimes a seasonal cycle is selected, in order to replicate the time of year when the organisms are collected from the wild, such as summer with a ligh:dark cycle of 16:8 h (Weber et al., 2018) or autumn with a cycle of 10:14 h (Garcia-Galan et al., 2017), (Table 2.1). Adopting different light:dark cycles could make the comparison between studies

challenging, since light could influence the organisms' physiological processes and behaviour (Perrot-Minnot et al., 2013).

Table 2.1. Existing differences in the literature regarding *Gammarus* spp. acclimation conditions.

Test organism	Age/Sex/Size organism	Duration	Temperature	Light:Dark Cycle	Type of water Aeration pH	Type of study	References
Gammarus fossarum	Free from parasites No gravid females	7 days	16°C	12:12	Mixed water Aerated	Feeding and assimilation study	(Blarer & Burkhardt-Holm, 2016)
Gammarus fossarum	Adults with a cephalothorax length between 1.2-1.6 mm	7 days	15°C		River water	Feeding preferences study	(Bundschuh et al., 2009)
Gammarus fossarum	Conducted as described by (Bundschuh et al., 2009)					Feeding rate study	(Bundschuh et al., 2011a)
Gammarus fossarum	Free from parasites Adults with a cephalothorax length between 1.2-1.6 mm	7 days	15°C		River water	Feeding rate study	(Bundschuh et al., 2011b)
Gammarus fossarum	Free from parasites No gravid females Adults with a cephalothorax length between 1.2-1.6 mm	7 days	15°C		River water	Feeding rate study	(Bundschuh et al., 2013)

Test organism	Age/Sex/Size organism	Duration	Temperature	Light:Dark Cycle	Type of water Aeration pH	Type of study	References
Gammarus fossarum	Adults with a cephalothorax length between 1.2-1.6 mm	7 days	15°C	Total darkness	River and tap water mixture	Feeding behavioural study	(Bundschuh et al., 2017)
Gammarus fossarum	Juveniles and adult males	15 days	12°C	10:14	Groundwater mixed with osmosed water Aerated	*Ex-situ and In-situ* feeding assay	(Coulaud et al., 2011)
Gammarus fossarum	Dry mass= 6.8 ± 0.7 mg	7 days	10°C			Decomposition and feeding rate study	(Danger et al., 2012)
Gammarus fossarum		20-25 days	12°C	10:14	Drilled groundwater Aerated	*In-situ* feeding experiment	(Dedourge-Geffard et al., 2009)
Gammarus fossarum	Adult males	21 days	12°C	10:14	Ground water Aerated	Bioaccumulation study	(Garcia-Galan et al., 2017)
Gammarus fossarum	Adult females	30-35 days	12°C	16:08	Drilled groundwater Aerated	Reproductive cycle and feeding study	(Geffard et al., 2010)
Gammarus fossarum	Adult males with diameter from 1.6-2.0 mm	7 days	16°C	Total darkness	SAM-5S medium	Feeding rate study	(Newton et al., 2018)

Test organism	Age/Sex/Size organism	Duration	Temperature	Light:Dark Cycle	Type of water Aeration pH	Type of study	References
Gammarus fossarum		10 days	12°C	8:16	Drilled groundwater Aerated	Feeding behaviour and biomarkers analysis	(Xuereb, et al., 2009)
Gammarus fossarum	Free from parasites Adults with a cephalothorax length between 1.2-1.6 mm	7 days	15°C		River water Aerated	Feeding, accumulation and growth study	(Zubrod et al., 2010)
Gammarus fossarum	Free from parasites Adult males (6-8 mm)	7 days	20°C	Total darkness	Aerated medium	Feeding and survival study	(Zubrod et al., 2014)
Gammarus fossarum	Free from parasites Adult males (6-8 mm)	7 days	16°C	Total darkness	SAM-5S medium Aerated	Toxicity and feeding study	(Zubrod et al., 2015)
Gammarus fossarum	Free from parasites Different sizes	3 days	16°C		SAM-5S medium Aerated	Feeding behavioural and physiological responses	(Zubrod et al., 2017)

Test organism	Age/Sex/Size organism	Duration	Temperature	Light:Dark Cycle	Type of water Aeration pH	Type of study	References
Gammarus pseudolimnaeus	Juveniles and adults					Feeding behavioural study	(Bärlocher & Kendrick, 1973b)
Gammarus pulex	Free from parasites Dry body mass 3.8-15 mg	3 days	13 °C	12:12	Artificial pond water	Feeding rate study	(Agatz et al., 2014)
Gammarus pulex	Organisms with parasites Both sexes Juveniles and adults	1 day	13°C	12:12	Artificial pond water Aerated pH = 7.4-7.9	Feeding rate studies	(Agatz & Brown, 2014)
Gammarus pulex	Free from parasites Adults (mean size 9,7 ± 1,4 mm) No gravid females	**1.** 4 days **2.** 4 days	**1.** 15° **2.** 20°C		River water Artificial water Aerated	Feeding rate study with the Multispecies Freshwater Biomonitor	(Alonso et al., 2009)
Gammarus pulex	3 - 7 mm		13°C	12:12	Dechlorinated tap water pH = 7.7	Feeding behavioural study	(Blockwell et al., 1998)
Gammarus pulex	Adult males	7 days	15°C	12:12		*In-situ* feeding assay	(Crane & Maltby, 1991)

Test organism	Age/Sex/Size organism	Duration	Temperature	Light:Dark Cycle	Type of water Aeration pH	Type of study	References
Gammarus pulex	Males with first thoracic segment of 0.7-1.2 mm in size	7 days	15°C	12:12	River water	Feeding behavioural study	(De Castro-Català et al., 2017)
Gammarus pulex	Adults (7-9 mm)	10 days	12°C		Well water pH = 7.19 ± 0.02	Physiological and behavioural responses	(Felten et al. 2008a)
Gammarus pulex	Free from parasites Adult males (dry weight 6.5-12.0 mg)		15°C	12:12	Artificial pond water	*In-situ* and laboratory feeding studies	(Forrow & Maltby, 2000)
Gammarus pulex	Adults (dry weight 8-10 mg)					Feeding behavioural study	(Graça et al. 1993a)
Gammarus pulex	Adults (9-10 mm) Juveniles (2.5-3.5 mm)		15°C	12:12	Artificial pond water	Feeding behavioural study	(Graça et al. 1993b)
Gammarus pulex	Adults		13°C			Feeding behavioural study	(Hahn & Schulz, 2007)
Gammarus pulex	Wet weight= 1.5-2.5 mg	10 days			Dechlorinated city tap water	Growth and feeding rate study	(Hargeby & Petersen, 1988)
Gammarus pulex	Both sexes		14°C	12:12	River water	Energetic state study	(Iltis et al., 2017)

Test organism	Age/Sex/Size organism	Duration	Temperature	Light:Dark Cycle	Type of water Aeration pH	Type of study	References
Gammarus pulex	Males (13-16 mm)		12°C	14:10	Aerated	Predation behaviour study	(Kelly et al., 2002)
Gammarus pulex	Adults	7-14 days	19 - 22°C		Dechlorinated tap water Aerated pH= 8.28 ± 0.06	Feeding and bioaccumulation study	(Lahive et al., 2015)
Gammarus pulex	Adult males (mean dry weight = 8.24 mg)	5-10 days	15°C	12:12	Artificial pond water	*In situ* feeding assay	(Maltby et al., 2002)
Gammarus pulex	Adult males (dry weight = 7-10 mg)	7 days	15°C	12:12	Artificial pond water	Scope for growth assay	(Naylor et al., 1989)
Gammarus pulex		1 day	14°C	16:8		Feeding behavioural study	(Taylor et al., 1993)
Gammarus pulex	Adults Juveniles	7 days	16°C	16:8	ISO medium Aerated	Feeding activity and physiological responses	(Weber et al., 2018)
Gammarus roeselii	Both sexes		15°C	12:12	Lake water	Feeding, assimilation and growth study	(Gergs & Rothhaupt, 2008)
Gammarus spp.		7 days			River water Aerated	Selective feeding study	(Arsuffi & Suberkropp, 1989)

Test organism	Age/Sex/Size organism	Duration	Temperature	Light:Dark Cycle	Type of water Aeration pH	Type of study	References
Gammarus spp.		5 days	10°C		River water pH = 7.2	Physiological and behavioural responses	(Maul et al., 2006)

2.3.4 Media selection

The type of media selected for an experiment is another factor that could have an impact on the outcome of a study. Some researchers prefer to use an artificial medium (Table 2.1) that guarantees standardisation (Agatz et al., 2014; Maltby et al., 2002), and in other studies, river water is sometimes used as a medium. However, river water might be contaminated, and this could therefore interfere with the organisms' cleansing process during their acclimation period, which makes it a peculiar choice of test media. Numerous studies have also used river water or a mixture (Alonso et al., 2009; Blarer & Burkhardt-Holm, 2016; Bundschuh et al., 2009, 2017; De Castro-Català et al., 2017; Dedourge-Geffard et al., 2009; Gergs & Rothhaupt, 2008; Iltis et al., 2017; Maul et al., 2006; Zubrod et al., 2015) (Table 2.1). For example, Bundschuh et al., (2017) combined river water with tap water, which also has limitations as the tap water could be contaminated (Magi et al., 2018). Potentially, any type of water could be contaminated, which is why the authors recommend that researchers should report the chemical breakdown (i.e. presence of contaminants) of their chosen water media along with their study findings so that any contamination is transparent.

Gammarus pulex allocates up to 11% of its energy supply to osmotic regulation (Sutcliffe, 1984), and Gammarids have been proven to be acid-sensitive (*Gammarus fossarum*; Felten & Guerold, 2001; *Gammarus pulex*, Sutcliffe & Carrick, 1973). In fact, acidic conditions induce a range of physiological and behavioural alterations, such as a reduction in the ventilation activity of *Gammarus pulex* (Felten et al., 2008b). These findings highlight the importance of measuring pH, as a shift in pH might influence the outcome of an experiment and prevent comparisons between studies. pH is rarely reported and presumably not measured in the environment during the collection process, the acclimation period or the experiment. Along with the chemical parameters of the acclimation media, the authors also recommend that pH is another factor that should be measured during the acclimation period, to ensure that accurate baseline data are recorded.

2.3.5 Characteristics of the test organism

Another important factor that plays a fundamental role in the reproducibility of a feeding experiment is the organism itself. Organisms of different age and sex may behave or respond differently to contaminants. For example, juveniles are more sensitive to contaminants than adult organisms (Adam et al. 2010), and their feeding rate varies over time, making them more suitable for short-term feeding studies (Agatz & Brown, 2014). Agatz & Brown (2014) and Nilsson (1974) identified that smaller specimens of *Gammarus pulex* have a higher feeding rate but higher variability over time, in comparison to larger organisms.

However, other studies have reported that the feeding rate increases with organism size (Coulaud et al., 2011), but the adoption of different units of measurement and a small size range might be contributing factors for those findings. It has been suggested that using organisms with a specific body mass (given in dry weight) could reduce experimental variability. For feeding studies, up to a 57% reduction in variability has been documented for specific body mass studies compared to mixed body mass studies (Agatz & Brown, 2014). There is also a recommendation that body length should be used as an indicator of dry weight and the correlation for organisms between 2 and 16 mm (Graça et al., 1993b).

Alternatively, organisms might be divided into size groupings by applying passive underwater separation techniques (Bundschuh et al., 2009, 2017; Zubrod et al., 2017), by measuring the dorsal length of the Gammarids' first thoracic segment after the organisms are photographed (De Castro-Català et al., 2017), by considering their wet weight (Blockwell et al., 1996; Danger et al., 2012; Weber et al., 2018), or by using their dry weight at the end of an experiment (Agatz et al., 2014). There is no agreed standard method on how to separate or select specimens of Gammarids for this experimental technique, but the chosen method will ultimately determine the unit of measurement for calculating the feeding rate, for example if wet weight is used, the unit of measurement will be wet weight. The use of either dry or wet weight seems straightforward, but it is only an estimate, and it lacks accuracy, as the dry weight range is only known at the end of the study. Furthermore, wet weight does not provide an accurate measurement due to the unknown volume of liquid in each sample. Blotting the sample dry

before weighing could help to remove a proportion of the moisture, but it could potentially stress the organisms and consequently affect the results; therefore, the authors recommend the use of dorsal length as the authors believe it to be a more accurate way to measure the organisms. In *in situ* experiments, the organisms are often divided by size before the start, but the weight is not taken into consideration. This means that the amount of consumed food is usually related to the number of living organisms at the end of the experiment (e.g. Coulaud et al., 2011; Dedourge-Geffard et al., 2009) (Table 2.3).

Same-sex tests with organisms (female-only, Geffard et al., 2010, or male-only, Crane & Maltby, 1991; De Castro-Català et al., 2017; Forrow & Maltby, 2000; Kelly et al., 2002; Maltby et al., 2002; Naylor et al., 1989; Zubrod et al., 2015) (Table 2.1) of a specific size are often undertaken, although sex is not always specified, which leads to female and male organisms being used indiscriminately (Agatz et al., 2014; Agatz & Brown, 2014; Alonso et al., 2009; Arsuffi & Suberkropp, 1989; Bärlocher & Kendrick, 1973a; Blarer & Burkhardt-Holm, 2016; Blockwell et al., 1998; Bundschuh et al., 2009, 2011b, 2013, 2017; Dedourge-Geffard et al., 2009; Gergs & Rothhaupt, 2008; Graça et al., 1993a, b; Hahn & Schulz, 2007; Lahive et al., 2015; Taylor et al., 1993; Weber et al., 2018; Xuereb et al., 2009; Zubrod et al., 2017) (Table 2.1).

As a rule, and not only in feeding studies, gravid females and organisms affected by the acanthocephalan parasite are usually excluded from experiments (Agatz et al., 2014; Alonso et al., 2009; Blarer & Burkhardt-Holm, 2016; Bundschuh et al., 2011b, 2013; Forrow & Maltby, 2000; Zubrod et al., 2015, 2017) unless they are specifically chosen for the purpose of the study (Agatz & Brown, 2014; Pascoe et al., 1995). Alonso et al., (2009) developed a feeding study using the Multispecies Freshwater Biomonitor (MFB), and neither length nor sex influenced the feeding activity of either sex of *Gammarus pulex*. However, it is debatable whether these results might only be applicable to the type of contaminant used in the investigation, as some contaminants might affect male and female Gammarid feeding behaviour in different ways.

2.4 Food preparation

The food source selected for an experiment using Gammarids is important, but especially so for feeding studies, both in the acclimation period and in the experiment itself. Gammarids are shredder detritivores, and they usually feed on conditioned organic material, in other words material that has been colonised by microorganisms, such as leaf litter. In the natural environment, freshly abscised leaves are colonised by fungi and then by bacteria (Baldy et al., 1995), which facilitate the decomposition process and transform the material, making it more palatable and accessible to the organisms (Bärlocher & Kendrick, 1975; Cummins, 1974; Gessner et al., 1999).

Gammarus spp. have displayed selective behaviour towards leaf species and their conditioning level (Agatz & Brown, 2014; Graça et al., 1993a, b, 2001) and the type of fungi (Arsuffi & Suberkropp, 1989). Interestingly, Graça et al. (2001) compared food preferences of shredders from temperate (*Gammarus pulex* and *Sericostoma vittatum*) and tropical (*Nectopsyche argentata* and *Phylloicus priapulus*) streams. When provided with conditioned and/or unconditioned leaves from either a temperate (*Alnus glutinosa*) or tropical (*Hura crepitans*) country, *Gammarus pulex* showed a significant preference for the conditioned leaves compared to unconditioned leaves of the same species. Leaves are characterised by different hardness, texture and more importantly by dissimilar C:N ratios, which means the various leaf species provide the organisms with differing energy supplies. A lower C:N ratio signifies a better quality food, and conditioned material is usually characterised by a lower C:N ratio compared to unconditioned material (Graça et al., 1993b). Some species such as alder (*Alnus* spp.) are characterised by a lower C:N ratio and higher palatability compared to others, such as horse chestnut (*Aesculus* spp.) (Agatz & Brown, 2014), which could lead to the organisms growing larger (Bärlocher & Kendrick, 1973b).

In feeding assays, there are several options when considering a food source. The most common choice is to provide the Gammarids with conditioned organic material. Depending on the study, the adopted leaf species may be different. The most commonly used leaves are alder (*Alnus* spp.), elm (*Ulmus* spp.), horse chestnut (*Aesculus* spp.), maple (*Acer* spp.), poplar (*Populus* spp.) and oak (*Quercus* spp.), (Table 2.2). In some cases, the Gammarids' diet is enriched with

Tubifex worms (Coulaud et al., 2011; Dedourge-Geffard et al., 2009; Geffard et al., 2010; Xuereb et al., 2009). Occasionally, they are provided with other types of food, such as alimentary chips (Novo Crabs®, JBL GmbH & Co., Germany), (Foucreau et al., 2014), Chironomidae (Gergs & Rothhaupt, 2008), *Artemia salina*'s eggs (Blockwell et al., 1998; Pascoe et al., 1995; Taylor et al., 1993), industrial shrimp food (Henry et al., 2017), fish food (Semsar-kazerouni & Verberk, 2018) or ground and tropical fish food mix (Blockwell et al., 1996).

During the acclimation period, organisms are normally fed *ad libitum* with pre-prepared conditioned leaves (Blarer & Burkhardt-Holm, 2016; Blockwell et al., 1998; Bloor, 2010; Bundschuh et al., 2011b; Crane & Maltby, 1991; Dedourge-Geffard et al., 2009; Geffard et al., 2010; Naylor et al., 1989; Newton et al., 2018; Xuereb et al., 2009; Zubrod et al., 2015). The conditioning process can vary, and the differences between the techniques can be found in Table 2.2.

In behavioural studies, food is supplied to the organism during the testing regime and is usually the same food type as provided during the acclimation period. The type of food used in a study could influence the feeding activity, especially if the organisms are fed on leaves that are not palatable or with leaves that have dissimilar energy budgets (e.g. Agatz & Brown, 2014).

Sometimes leaves are collected at the beginning or during fall, specifically handpicked senescent *Alnus glutinosa* leaves that are not decomposed (Bundschuh et al., 2009, 2017), whereas in other studies, the leaves are specifically collected after they had abscised (Hargeby & Petersen, 1988). After collection the leaves are either used straight away or stored for later use (Table 2.2). Storage methods vary throughout the literature, for example, Bundschuh et al. (2009, 2011a, b, 2013, 2017) froze their leaves at -20°C, but this methodology ultimately alters the structure of the leaves (Burke et al., 1976). More commonly, the leaves are dried at room temperature and stored in the dark until needed (e.g. Naylor et al., 1989) (Table 2.2). However, Gessner et al. (1999) highlighted that drying leaves in an oven or at room temperature ultimately ruins the leaf tissue. In the natural environment, leaves usually reach water bodies soon after abscission (Fisher, 1977). Consequently, storing leaves for later use does not mimic the natural chain of events, and storing will ultimately disrupt their structure. Gessner and Schwoerbel (1989) demonstrated that freezing or drying leaves

increases mass loss in the first few days when in water, and this accelerates the conditioning process, which is usually statistically delayed in fresh leaves (Bärlocher, 1992).

The conditioning process involves soaking the leaves in water and mixing them with an unknown fungi species (Nilsson, 1974) or by inoculating the leaves with a specific fungi species (Naylor et al., 1989). In the first instance, river water might be used in the laboratory to condition the leaves, and it is usually inoculated with organic material taken directly from the river as a natural source of fungi and bacteria (e.g. Zubrod et al., 2015, 2017), (Table 2.2). Leaves can also be directly conditioned *in situ* by placing them in small nets/bags that are suspended in a river and retrieved after a specific number of days (Alonso et al., 2009; De Castro-Català et al., 2017; Forrow and Maltby, 2000; Graça et al., 2001; Zubrod et al., 2015), (Table 2.2).

Although river water might reproduce natural environmental conditions, it is sometimes contaminated, and this might have an impact. When river water is used, a chemical breakdown of the water should be undertaken and reported along with the study findings, so that any contamination is transparent. It is especially important to disclose if the river water is contaminated with the substance(s) under investigation in the study. If the test substances are present in the river water, the organism could be exposed to that concentration and also the experimental dose. Therefore, the organisms' responses would not be a true reflection of the test concentration(s) but instead the reported dose combined with the concentration found in the river water. For example, contaminants might be absorbed onto the leaf surface and passed onto the organisms, or they could be released into the media, which might happen during the acclimation period and/or during the experiment itself, resulting in a compromised feeding activity. Therefore, the observed findings might be an indirect effect, due to the leaf quality and not as a direct result of the contaminant being tested.

The conditioning process usually takes around 2 weeks, but there are clear differences in the literature about this stage (Table 2.2). The process ranges from a few days (Alonso et al., 2009), to several weeks (Blarer & Burkhardt-Holm, 2016), and up to months (Danger et al., 2012), (Table 2.2). When *Gammarus* spp. are offered a choice between leaves that have been conditioned for different

periods of time, they prefer those that have been conditioned for the longest (Agatz & Brown, 2014; Bird & Kaushik, 1985). Consequently, experiments (*in situ* or *ex situ*) that provide the organisms with leaves that have been conditioned for a short or longer time period could potentially underestimate or overestimate the actual feeding activity of *Gammarus* spp.

It has been demonstrated that conditioned leaf material is more palatable (Agatz & Brown, 2014; Graça et al., 1993b) and that different species of leaves (i.e. *Acer* spp. and *Ulmus* spp.), depending on the conditioning stage, might be more or less palatable compared to the others (Bird & Kaushik, 1985). Consequently, it could be argued that it is impossible to compare experiments where organisms have been fed with organic material that has been conditioned for different periods of time. Organisms fed on leaves that have been conditioned for 1 week will probably eat less than those fed with the same leaves conditioned for 3 weeks, and leaf unpalatability might be mistakenly attributed to contaminant exposure. It has also been identified that *Gammarus pulex* fed with unconditioned leaves have a considerably lower respiration rate (Graça et al., 1993b).

Depending on the methodology used, the conditioning process might take place in different phases. There are studies where the leaves are provided to the organisms directly after the conditioning process (Bärlocher & Kendrick, 1973a; Bloor, 2010; Bundschuh et al., 2009; Forrow & Maltby, 2000; Newton et al., 2018), whereas in some cases the leaves are redried and soaked in water before feeding them to the organisms (Agatz et al., 2014; Blarer & Burkhardt-Holm, 2016; Bundschuh et al., 2011b, 2013, 2017; Naylor et al., 1989), in order to prevent them from floating on the surface. In this case, the drying process requires the use of an oven, but unsurprisingly the time and temperature used vary between research groups. Bear in mind that the same food might be provided during the acclimation period and also during the experiment itself, unless the feeding experiment aims to study the feeding variation when a food source is either contaminated or compromised. In these studies, a specific contaminant or mixture of contaminants are usually incorporated during the conditioning process (Bundschuh et al., 2009; Hahn & Schulz, 2007). When the conditioned leaves are oven-dried, they need to be resoaked in water before being provided to the organisms, in order to avoid floatation (e.g. Bundschuh et al., 2017; Zubrod et al., 2010). The water used to soften the leaves varies between research groups, and

the water could act as a new source of contamination, especially if it differs from the one used during the original conditioning process.

Table 2.2. Existing differences in the literature regarding the conditioning process.

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
Acer saccharum *Ulnus spp*.	Cut in discs of 9 mm diameter, leached in tap water for 4 days at 12°C, and dried at 40°C for 2 days. Stored at room temperature.	**Experiment food:** Nutrient enriched river water	*Alternaria* sp. *Fusarium* sp. *Cladosporium* sp. *Humicola grisea* *Aspergillus niger* *Tricladium angulatum* *Tetracladium marchalianum* *Anguillospora longissima Clavariopsis aquatica* *Flagellospora curvula*				Feeding study	(Bärlocher & Kendrick, 1973b)
Acer saccharum	Air-dried and stored in plastic bags until use.	**Experiment food:** River water		4 days	After being cut in leaf discs of 1.1 cm in diameter and being dried for 3 days at 60°C.		Physiological and behavioural responses	(Maul et al., 2006)
Aesculus hippocastanum	**Acclimation food**: Stored in tap water for 3 months	**Acclimation food**: Tap water	*Cladosporium* spp.	10 days		*Ad libitum*	Feeding study	(Agatz et al., 2014)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
	Experiment food: dried at room temperature	**Experiment food:** Enriched water			**Experiment food:** Before re-drying at 60˚C			
1. *Aesculus hippocastanum* 2. *Alnus glutinosa*	**Acclimation food**: Whole horse chestnut leaves stored in tap water **Experiment food:** **1.** Air dried in the dark at room temperature (20°C)	**Acclimation food**: Tap water **Experiment food:** 1. Enriched water or tap water 2. Nutrient medium	**Acclimation food**: *Cladosporium* spp. **Experiment food:** 1. *Cladosporium* spp.	**Acclimation food**: 3 months **Experiment food:** 1. 2 weeks or 3 months 2. 10 days	**Experiment food:** 2. After being cut in discs of 2.0 cm in diameter, but before re-drying at 60˚C		Feeding study	(Agatz & Brown, 2014)
Aesculus hippocastanum		**Acclimation food**: conditioned in organically enriched dechlorinated water (Bird & Kaushik, 1985)		10 days		*Ad libitum*	Feeding study	(Blockwell et al., 1998)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
Aesculus hippocastanum		Organically enriched water, following method of (Bird & Kaushik, 1985)		10 days			Feeding study	(Taylor et al., 1993)
Alnus glutinosa	Air dried for 1 h and then stored at -20°C.	**Acclimation food:** Decaying leaves collected in a pond. **Experiment food:** Cut in leaf discs of 2 cm in diameter and conditioned directly in the river		21 days	After being cut in leaf discs of 2 cm in diameter, but before been dried at 60°C for 24h. Soaked for 24h in water before being used in the experiment.	**Acclimation food:** *Ad libitum*	Feeding and assimilation study	(Blarer & Burkhardt-Holm, 2016)
Alnus glutinosa	Froze at -20°C until use	**Experiment food:** Soaked in tap water for 24h and then conditioned in a nutrient medium with added leaf litter from the river		From 19 to 22 days	After being cut in leaf discs of 1.5 cm in diameter and been dried at 60°C for 24h		Feeding preferences study	(Bundschuh et al., 2009)
Alnus glutinosa	Frozen at -20°C and stored until use. Following the method described by (Bundschuh et al., 2009)	**Acclimation food:** Preconditioned leaves **Experiment food**:		12 days	After being cut in leaf discs of 1.6 cm in diameter and dried.		Feeding preferences study	(Bundschuh et al., 2011a)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
		Conditioning medium (Dang, et al., 2005) inoculated with leaves previously conditioned directly in the river						
Alnus glutinosa	Froze at -20°C until use	**Acclimation food:** Pre-conditioned Alder leaves **Experiment food:** Conditioned in a nutrient medium with added leaves that were previously conditioned directly in the river		10 days	After being cut in leaf discs of 2 cm in diameter, but before been dried at 60°C for 24h	Pre-conditioned alder leaves fed *ad libitum*	Feeding study	(Bundschuh et al., 2011b)
Alnus glutinosa	Froze at -20°C until use	**Acclimation food:** Pre-conditioned Alder leaves **Experiment food:** Conditioned in a nutrient medium with added leaves that were previously		10 days	After being cut in leaf discs of 2 cm in diameter, but before been dried at 60°C for 24h		Feeding study	(Bundschuh et al., 2013)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
		conditioned directly in the river						
Alnus glutinosa	Froze at -20°C until use	**Acclimation food:** Pre-conditioned Alder leaves **Experiment food:** Conditioned in a mixture of tap water and stream water with pre-conditioned leaves coming from the river.		18 days	Before being cut in leaf discs of 2 cm in diameter and been dried at 60°C for 24h. Soaked for 24h in tap water before being used in the experiment.		Feeding study	(Bundschuh et al., 2017)
Alnus glutinosa		**Acclimation and Experiment food:** groundwater Freeze-dried *Tubifex* worms added to the food twice a week.		6 days		*Ad libitum*	Laboratory and *In situ* feeding study	(Coulaud et al., 2011)
Alnus glutinosa			*Cladosporium*			*Ad libitum*	*In situ* feeding study	(Crane & Maltby, 1991)
Alnus glutinosa		**Experiment food:** Conditioned in a river water.		14 days			Feeding study	(De Castro-Català et al., 2017)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
Alnus glutinosa		**Acclimation and Experiment food:** Conditioned in water Freeze-dried *Tubifex* worms added to the food twice a week.		6 days		**Acclimation food:** *Ad libitum*	*In situ* feeding study	(Dedourge-Geffard et al., 2009)
Alnus glutinosa	Air-dried and stored at room temperature until use	**Acclimation food:** Fungally conditioned leaves. **Experiment food:** Two different types depending on the experiment. 1. Leaves were naturally conditioned in the river 2. Method described by	*2. Cladosporium*		Before being cut in leaf discs of 1 cm in diameter and been blotted dry and weighed. Used immediately afterwards.		*In situ* and laboratory feeding studies	(Forrow & Maltby, 2000)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
		(Naylor et al., 1989)						
Alnus glutinosa		**Acclimation food:** Conditioned in water Freeze-dried *Tubifex* worms added to the food twice a week.		6 days		**Acclimation food:** *Ad libitum*	Reproductive cycle and feeding study	(Geffard et al., 2010)
Alnus glutinosa		**Experiment food:** Naturally conditioned in the lake		21 days			Feeding, assimilation and growth study	(Gergs & Rothhaupt, 2008)
Alnus glutinosa *Hura crepitans*	Air-dried and stored until use.	**Experiment food:** Different types. Depending on the experiment. 1. Conditioned directly into the river		1. 14 days 2. 24 h	Before being cut in leaf discs of 1.4 cm in diameter.		Feeding study	(Graça et al., 2001)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
		2. Leaves soaked in tap water						
Alnus glutinosa	Frozen at -20°C until use	**Experiment food:** River water		14 days	After being cut in leaf discs of 2 cm in diameter.		Feeding study	(Hahn & Schulz, 2007)
Alnus glutinosa		**Acclimation and Experiment food:** Method described by (Naylor et al., 1989)					*In situ* feeding study	(Maltby et al., 2002)
Alnus glutinosa	Dried and stored	**Acclimation and Experiment food:** Enriched water	*Cladosporium* spp.	10 days	After being rehydrated, cut in leaf discs of 1.6 cm in diameter and autoclaved, but before being dried for 2 days at 60°C. Rehydrated again before being fed.	**Acclimation food:** *Ad libitum*	Scope for growth assay	(Naylor et al., 1989)
Alnus glutinosa	Froze at -20°C.	**Acclimation food:** Preconditioned leaves		1. 13 days 2. 14 days	1. After being cut in leaf discs of 1.6 cm in diameter, being froze for 24 and	**Acclimation food:** *Ad libitum*	Feeding study	(Newton et al., 2018)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
		Experiment food: 1. Conditioning medium as (Dang et al., 2005) inoculated with alder leaves conditioned in the river for 14 days 2. Conditioning medium			subsequently weighed. Unconditioned leaves either submerged for 2 min or 48 h in SAM-5S medium 2. Before being cut in leaf discs of 2.0 cm in diameter and being directly fed to the organisms			
Alnus glutinosa *Fagus silvatica*	Dried at 20°C.	**Experiment food:** River water		10 days			Feeding, assimilation and respiration study	(Nilsson, 1974)
Alnus glutinosa		**Acclimation and Experiment food:** Conditioned in water Freeze-dried *Tubifex* worms		6 days		**Acclimation food:** *Ad libitum*	Feeding behaviour and biomarkers analysis	(Xuereb et al., 2009)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
		added to the food twice a week.						
Alnus glutinosa	Frozen at -20°C and stored until use. Following the method described by (Bundschuh et al., 2011b).	**Acclimation food:** Preconditioned leaves **Experiment food**: Conditioning medium (Dang et al., 2005) inoculated with leaves previously conditioned directly in the river		10 days	After being cut in leaf discs of 2.0 cm in diameter, but before being dried at 60°C for 24h and weighed. Soaked for 24h in tap water before being used in the experiment.	**Acclimation food:** *Ad libitum*	Feeding, accumulation and growth study	(Zubrod et al., 2010)
Alnus glutinosa	Frozen at -20°C and stored until use. Following the method described by (Zubrod et al., 2010)	**Acclimation food:** Preconditioned leaves **Experiment food**: Conditioning medium (Dang et al., 2005) inoculated with leaves previously conditioned directly in the river		10 days	After being cut in leaf discs of 2.0 cm in diameter, but before being dried at 60°C for 24h and weighed. Soaked for 48h in tap water before being used in the experiment.	**Acclimation food:** *Ad libitum*	Feeding an survival study	(Zubrod et al., 2014)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
Alnus glutinosa	Frozen at -20°C and stored until use.	Conditioned in medium with leaves that were previously conditioned directly in the river for 14 days.		12 days		**Acclimation food:** *Ad libitum*	Toxicity and feeding study	(Zubrod et al., 2015)
Alnus glutinosa	Frozen at -20°C and stored until use.	Conditioned in stream water with leaves that were previously conditioned directly in the river for 14 days and for 14 days in the lab.		13 days			Feeding Behaviour and Physiological responses	(Zubrod et al., 2017)
Alnus spp.	Air dried and stored	River water with detritus		At least 10 days	After being air dried	*Ad libitum*	Laboratory breeding program.	(Bloor, 2010)
Fraxinus pennsylvanica *Acer saccharum* *Quercus velutina*	Cut in discs of 1-2 cm diameter, leached in tap water for 4 days at 12°C, and dried at 40°C for 2 days. Stored at room temperature in polyethylene bags.		*Alternaria* spp. *Fusarium* spp. *Cladosporium* spp. *Aspergillus niger* *Humicola grisea* *Tricladium angulatum* *Tetracladium marchalianum*	14 days	After drying			(Bärlocher & Kendrick, 1973a)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
			Anguillospora longissima *Clavariopsis aquatica* *Flagellospora curvula*					
Populus sp		**Acclimation food**: conditioned naturally in the river **Experiment food:** mixture of stream water and Dutch Standard Water – DSW		**Acclimation food**: **Experiment food:** 4 days			Feeding rate study with the Multispecies Freshwater Biomonitor	(Alonso et al., 2009)
Populus tremuloides		**Experiment food:** Incubation medium Stream water	*Flagellospora curvula* *Alatospora acuminata* *Clavariopsis aquatic* *Tetracladium marchalianum* *Lemonniera aquatica* *Heliscus lugdunensis*	Either 10 or 15 days depending on the fungi and then 72 h in stream water	**Experiment food:** Before drying at 45°C		Selective feeding study	(Arsuffi & Suberkropp, 1989)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
			Articulospora inflata *Filosporella annelidica*					
Quercus petraea		**Experiment food:** Conditioned directly into the stream		35, 56 or 82 days	Before being cut in leaf discs of 1.2 cm in diameter and being frozen at -18°C	**Acclimation food:** *Ad libitum* with plant detritus from the stream.	Decomposition and feeding study	(Danger et al., 2012)
Ulmus caprinifolia	Air dried and leached for 96h in distilled water and air dried again.	**Acclimation food:** Decaying leaves **Experiment food:** Incubated in soft water		**Experiment food:** 2 to 3 weeks	Before being given to the organisms	**Acclimation food:** *Ad libitum*	Growth study	(Hargerby & Petersen, 1988)
Ulmus procera		**Experiment food:** Different types. Depending on the experiment. 1. Conditioned in stream water where leaf material from the river was added.		1. 14 days 2. 4 days 3. 15 days 4. 14 days	3. After being cut in leaf discs of 1.6 cm in diameter, but before drying at 60°C for 4 days. 4. After being cut in leaf discs of 1.6 cm in diameter and after being dried at 60°C for 4 days.		Feeding study	(Graça et al., 1993a)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
		2. Leaves soaked in tap water 3. Artificial pond water 4. Artificial pond water	*3. Anguillospora longissima* *Articulospora tetracladia* *Fusarium cavispermum* *Fusarium* spp. *Cylindrocarpon* spp. *Heliscus lugdunensis* *Lemonniera aquatica* *Tetracladium marchalianum* *Tetracladium setigerum* *Triclodium angulatum*					

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during Acclimation	Type of Study	Reference
			4. Anguillospora longissimi					
Ulmus procera		**Experiment food:** Different types. Depending on the experiment. **2.** Conditioned in artificial pond water where leaf material from the river was added. **3.** Leaves soaked in tap water		1. 21 days 2. 4 days	After being cut in leaf discs of 1.6 cm in diameter and after being dried at 60°C for 4 days.		Feeding study	(Graça et al., 1993b)

2.5 Exposure and feeding rate calculation

After the conditioning process and the acclimation period, the next step in a feeding study is the exposure itself. During this time, the Gammarids are exposed directly (i.e. the contaminant is in the water with the *Gammarus* spp.) (Zubrod et al., 2010) or indirectly (i.e. the contaminant is added during the conditioning process) (Bundschuh et al., 2009) to a contaminant, and their feeding behaviour is studied and estimated (Table 2.3). These experiments might have different goals: they might be undertaken to either measure the changes in Gammarid feeding activity, Gammarid feeding preferences, or to study the effects on their growth. Consequently, the period of exposure could vary dramatically from a few hours (Bundschuh et al., 2011a) to a week (Felten et al., 2008a) or even several weeks (Weber et al., 2018), and sometimes fungal biomass analysis (estimated as ergosterol) and assimilation are incorporated, to strengthen the findings obtained from the feeding rate (Bundschuh et al., 2009; Newton et al., 2018).

Occasionally in feeding studies, the organisms undergo a period of starvation before the experiment is undertaken (De Castro-Català et al., 2017) (Table 2.3). The main purpose of this starvation phase is to ensure that the organisms are at the same hunger state, but the duration of this phase varies in the literature. Once the experiment starts, the Gammarids are commonly provided with a precise amount of food, in other words the leaves provided have usually been dried, weighed and conditioned. This latter step, as previously mentioned, could have been carried out before the drying process or afterwards, so the final product could have different characteristics depending on the study. In order to provide the organisms with the same amount of food, the leaves are cut in small discs that range from a diameter of 0.7 to 4 cm depending on the research group (Table 2.3). Before or after the conditioning process, the leaf discs are oven-dried for a specific period of time, which is usually at the same temperature and for the same time period as used after the exposure (Table 2.3). Once the leaf discs have been weighed, they are usually resoaked in water or conditioned, if that is still to be done, and provided to the Gammarids during the experiment, after sometimes rinsing with water.

During and after the exposure, data are collected to calculate the feeding rate of the organisms. The feeding rate equation is similar throughout the literature, but

variations can still be found. For example, the data might not have been collected in the same way, even though the same equation might have been used. The most common way to estimate the feeding rate is to compare leaf dry weight before and after exposure to the amphipods, in relation to the duration of the experiment and the weight of the organisms. Commonly, the dry weight of the leaves is adjusted with a constant[4]. This constant takes the loss in weight due to leaching and microbial decomposition into consideration. It is often calculated as the ratio of the control leaves' final dry weight and their initial dry weight (e.g. Blarer & Burkhardt-Holm, 2016) (Table 2.3), but sometimes the equation might vary (e.g. Bundschuh et al., 2011b) (Table 2.3). The control leaves are leaf discs that went through the same conditioning process, and through the same experimental conditions as those fed to the organisms, but they themselves were not.

The constant is not always positioned in the same place within the feeding rate equation. Most commonly, it multiplies with the initial dry weight of the leaves (e.g. Maltby et al., 2002) (Table 2.3) as the initial dry weight might not be exact. A small amount of leaf might have been lost due to leaching and microbial decomposition during the conditioning process, for example. Sometimes the constant divides the final dry weight of the leaves (Agatz et al., 2014) (Table 2.3). A proportion of the leaf might have been lost through leaching and the decomposition process, and not through Gammarids consumption. Both constant positions are trying to adjust the equation by compensating for the same problem, leaching and decomposition, but mathematically the equations are dissimilar and the results might be different.

Weight is sometimes considered as wet weight (Danger et al., 2012) or as ash-free dry weight (AFDW) (De Castro-Català et al., 2017) rather the normal dry weight (Table 2.3). Once the exposure is complete, the leaf discs are collected and dried. The drying process is normally carried out in an oven and/or furnace (i.e. AFDW) at a specific temperature for a specific duration, which was also used for leaf disc preparation. As shown in Table 2.3, the temperature at which the leaves are dried can be very different and so can the duration of the process.

[4] From Chapter 3 onwards, the constant/leaching constant will be referred to as Leaf Change Correction Factor or simply Correction Factor (CF).

On rare occasions, the feeding rate is calculated by measuring differences in the leaf disc's surface area, which instead of being weighed are photographed and later analysed with a specific software (Coulaud et al., 2011; Hahn & Schulz, 2007) (Table 2.3). Scanning the leaf surface might result in very accurate data when it is calculated by pixel size or in mm^2, for example. This calculation does not incorporate a leaching constant (leaf change correction factor), which takes into account the loss of leaf weight due to the conditioning process. Differences in leaf surface could potentially occur as it happens with the loss in weight method. The authors acknowledge that it is still unclear if the choice of feeding equation and the different ways of calculating the feeding rate are actually comparable and equivalent. Interestingly Coulaud et al. (2011) reported a relationship between the surface and the dry mass of their leaf discs, in order to facilitate possible comparisons between studies with different methodologies. Consequently, it is recommended that a leaf change correction factor should be calculated based on leaf surface loss, to take leaf conditioning changes into consideration and to make data from these different techniques more comparable.

Table 2.3. Existing differences in the literature regarding feeding behavioural experiments.

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
Aesculus hippocastanum	Imidacloprid	Artificial pond water	12:12	1.6 cm 3 at the time and exchanged every 24 h	7 days (4 days exposure + 3 days recovery phase)	**Leaves**: **Before**: 60˚C for 48 h **After**: **Organisms**: **After**: 65˚C for 48 h		$FR = \frac{F[t-1]-\frac{F[t]}{ld}}{G*t}$ $FR = feeding\ rate$ $F[t-1] = initial\ leaf\ dry\ weight\ (mg)$ $F[t] = final\ leaf\ dry\ weight\ (mg)$ $G = dry\ weight\ of\ Gammarus\ (mg)$ $ld = leaching\ decomposition\ factor$ $t = time\ (days)$	(Agatz et al., 2014)
1. *Aesculus hippocastanum* 2. *Alnus glutinosa*		Artificial pond water	12:12	1. 1.6 cm 2. 2.0 cm	**Experiment 1**: 96h **Experiment 2**: 9 days **Experiment 3**: 15 days	**Leaves**: 1. **2. Before**: 60˚C to constant weight **After**: **Organisms**:		$FR = \frac{F[t-1]-\frac{F[t]}{ld}}{G*t}$ $FR = feeding\ rate$ $F[t-1] = initial\ leaf\ dry\ weight\ (mg)$ $F[t] = final\ leaf\ dry\ weight\ (mg)$ $G = dry\ weight\ of\ Gammarus\ (mg)$ $ld = leaching\ decomposition\ factor$ $t = time\ (days)$	(Agatz & Brown, 2014)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
						After: 90˚C for 24 h Wet weight transform in dry weight using a linear regression			
Alnus glutinosa	Microplastic debris	Mixed water (municipal water and softened water)		2.0 cm 2 discs at the time every 7 days	28 days	**Leaves**: **Before**: 60˚C for 24 h **After**: 60˚C for 24 h		$FR = \frac{L_i*(CF) - L_f}{w*t}$ $FR = feeding\ rate$ $L_i = initial\ leaf\ dry\ weight\ (mg)$ $L_f = final\ leaf\ dry\ weight\ (mg)$ $w = animal's\ average\ wet\ weight\ (mg)$ $t = feeding\ time\ (days)$ $CF = leaf\ change\ correction\ factor$ $CF = \frac{\left[\Sigma\left(\frac{C_f}{C_i}\right)\right]}{n}$	(Blarer & Burkhardt-Holm, 2016)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
								$C_f =$ *final dry weight of control leaf discs (mg)* $C_i =$ *initial dry weight of control leaf discs (mg)* $n = number\ of\ replicates$	
Alnus glutinosa	*In situ* and *ex situ* feeding assays	*In situ* : river water *Ex situ*: river water		*In situ*: 5 leaf discs per cage of 1.6 cm in diameter *Ex situ*: 5 leaf discs per pot of 1.6 cm in diameter	6 days	**Leaves**: **Before**: **After**: 60˚C for 4 days **Organisms**: **After**: 60˚C for 4 days	24 hours	$C = \frac{(L_1 * C_L) - L_2}{W * 6}$ $C = feeding\ rate$ $L_1 = initial\ leaves\ dry\ weight\ (mg)$ $L_2 = final\ leaves\ dry\ weight\ (mg)$ $W = organisms\ dry\ weight\ (mg)$ $C_L = leaf\ weight\ change\ correction\ factor$ $C_L = \frac{\Sigma\left(\frac{C_2}{C_1}\right)}{N}$ $C_1 = control\ leaf\ discs\ initial\ dry\ weight\ (mg)$	Bloor & Banks, 2006b

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
								$C_2 = control\ leaf\ discs\ final\ dry\ weight\ (mg)$ $N = number\ of\ control\ replicates$	
Alnus glutinosa	Antibiotics (added during the conditioning process)	River water	Total darkness	1.5 cm 4 leaves at the time to assess food choice	58 hours	**Leaves**: **Before**: 60˚C for 24 h **After**: 60˚C for 24 h **Organisms**: **After**: 60˚C for 24 h	96 hours	$C = \frac{\{[(f_b - f_a) - (n_b - n_a)] * 24\}}{g * t}$ $C = leaf\ mass\ consumed$ f_b = *initial dry mass of the leaf disc exposed to feeding (before conditioning) (mg)* f_a = *final dry mass of the leaf disc exposed to feeding (mg)* n_b = *initial dry mass of the leaf disc protected from feeding (mg)* n_a = *final dry mass of the leaf disc protected from feeding (mg)* g = *animal's dry weight (mg)* t = *feeding time (hours)*	(Bundschuh et al., 2009)
Alnus glutinosa	Fungicide tebuconazole			1.6 cm 4 leaf discs at the time, but only 2 discs were	12 h	**Leaves**: **Before**: **After**:			(Bundschuh et al., 2011a)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
				accessible to the organism		**Organisms**: **After**:			
Alnus glutinosa	Secondary treated Wastewater	River water or secondary treated wastewater		2.1 cm 2 leaves at the time every 7 days	4 weeks	**Leaves**: **Before**: 60˚C for 24 h **After**: 60˚C for 24 h **Organisms**: **After**: 60˚C for 24 h		$C = \frac{[L_b * (k) - L_e]}{g * t}$ $C = leaf\ mass\ consumed$ $L_b = initial\ dry\ mass\ of\ the\ leaf\ disc\ (mg)$ $L_e = final\ dry\ mass\ of\ the\ leaf\ disc\ (mg)$ $g = animal's\ dry\ mass\ (mg)$ $t = feeding\ time\ (days)$ $k = leaf\ change\ correction\ factor$ $k = \frac{\sum\left[\frac{L_{ob} - L_{oe}}{L_{ob}}\right]}{n}$ $L_{ob} = initial\ dry\ mass\ of\ control\ discs\ (mg)$ $L_{oe} =$ $final\ dry\ mass\ of\ control\ leaf\ discs\ (mg)$ $n = number\ of\ replicates$	(Bundschuh et al., 2011b)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
Alnus glutinosa	Antibiotics (added during the conditioning process and in the water with the Gammarids)	River and tap water mixture.		1.1 cm 2 leaves at the time	24 days	**<u>Leaves</u>**: **Before**: 60˚C for 24 h **After**: 60˚C for 24 h **<u>Organisms</u>**: **After**: 60˚C for 24 h		$C = \frac{[L_a*(1-k)-L_b]}{g*t}$ $C = leaf\ mass\ consumed$ $L_a =\ initial\ dry\ mass\ of\ the\ leaf\ disc\ (mg)$ $L_b = final\ dry\ mass\ of\ the\ leaf\ disc\ (mg)$ $g = animal's\ dry\ mass\ (mg)$ $t = feeding\ time\ (days)$ $k = \frac{\sum\left[\frac{L_c-L_d}{L_c}\right]}{n}$ $L_c = initial\ dry\ mass\ of\ control\ discs\ (mg)$ $L_d =$ $final\ dry\ mass\ of\ control\ leaf\ discs\ (mg)$ $n = number\ of\ replicates$	(Bundschuh et al., 2017)
Alnus glutinosa	*In situ* feeding assay	River water		1.7 cm 4 leaf discs per cage	6 days	**<u>Leaves</u>**: **Before**: **After**: 60˚C for 48 h **<u>Organisms</u>**:		$C = \frac{(L_1*C_1)-L_2}{W*T}$ $C = feeding\ rate$ $L_1 = initial\ leaves\ dry\ weight\ (mg)$	(Crane & Maltby, 1991)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
						After: 60˚C for 48 h		$L_2 = final\ leaves\ dry\ weight\ (mg)$ $W = organisms\ dry\ weight\ (mg)$ $C_1 = leaf\ weight\ change\ correction\ factor$ $T = feeding\ time\ (days)$	
Alnus glutinosa	*Ex situ* and *in situ* feeding assay	***Ex situ* experiment**: ***In situ* experiment**: River water	***Ex situ* experiment**: 10:14 ***In situ* experiment**:	2 .0 cm ***Ex situ*:** 20 discs per 20 gammarids ***In situ***: 20 discs for 20 gammarids	***Ex situ*:** 14 days ***In situ***: 7 days	**Leaves**: **Before** & **After**: Photo Scanned		$FR_i = \frac{(S_{control} - S_i)}{(\frac{l_{i,0}+l_{i,t}}{2})*t}$ $FR_i = feeding\ rate\ of\ replicate\ i$ $S_{control} = total\ surface\ of\ the\ control\ leaf\ discs\ at\ the\ end$ $S_i = total\ surface\ of\ the\ leaf\ disc\ at\ the\ end\ of\ replicate\ i$ $t = feeding\ time\ (days)$ $l_{i,0} =$ $number\ of\ living\ gamamrids\ at\ the\ start$ $l_{i,t} = number\ of\ living\ gamamrids\ at\ the\ end$	(Coulaud et al., 2011)
Alnus glutinosa *Quercus petraea*		Filtered river water		1.0 cm 2 leaf discs at the time, with just one accessible to the organsim	68 to 72 h	**Leaves**: **Before**: wet weight was used **After**: 65˚C			(Danger et al., 2012)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
						Organisms: **After**: 65˚C			
Alnus glutinosa	Antidepressant and fungicide (added to the leaves during the conditioning or in the water)	Filtered river water	12:12	1.3 cm	14 days	**Leaves**: Difference between initial and final ash-free dry mass (AFDW).	24 h		(De Castro-Català et al., 2017)
Alnus glutinosa	Metals: *in situ* experiment			2.1 cm 20 leaf discs at the time for each cage	14 days	**Leaves**: **Before** & **After**: Photo Scanned		$FR = \frac{\frac{(S_{i,t0} - S_{i,t7})}{\frac{l_{i,t0} + l_{i,t7}}{2}}}{\frac{\Sigma_{i=1}^{4}}{4}}$ $FR = 7$ $FR = feeding\ rate$ $i = (i = 1 - 4)\ is\ the\ ith\ replicate$ $S = total\ surface\ of\ leaf\ discs\ \ in\ each\ container\ (mm^2)$ $l = number\ of\ living\ gammarids$	(Dedourge-Geffard et al., 2009)
Alnus glutinosa	Cadmium	Well-water		2.0 cm	7 days	**Leaves**:		$FR = \frac{(L_f * C) - L_i}{WT}$	(Felten et al., 2008a)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
						Before: 105˚C for 24 h **After**: 105˚C for 24 h **<u>Organisms</u>**: **After**: 105˚C for 24 h		$FR = feeding\ rate$ $L_f = leaf\ final\ dry\ weight\ (mg)$ $L_i = leaf\ initial\ dry\ weight\ (mg)$ $W = organisms\ dry\ weight\ (mg)$ $T = exposure\ time\ (days)$ $C = leaching\ correction\ factor$ $C = \frac{\Sigma\left(\frac{L_b}{L_a}\right)}{8}$ $L_b = final\ dry\ weight\ of\ control\ leaves\ (mg)$ $L_a = initial\ dry\ weight\ of\ control\ leaves\ (mg)$	
Alnus glutinosa		***In situ***: river water **Lab experiments**: artificial pond water			***In situ***: 6 days or 12 days **Lab experiments**: 6 days	**<u>Leaves</u>**: **Before**: (just wet weight) **After**: 60˚C for 48 h **<u>Organisms</u>**: **After**: 60˚C for 48 h		$C = \frac{(L_b * C_L) - L_a}{W * T}$ $C = consumption\ of\ leaf\ material$ $L_b = leaf\ discs\ initial\ dry\ weight\ (mg)$ $L_a = leaf\ discs\ final\ dry\ weight\ (mg)$ $W = organisms\ dry\ weight\ (mg)$	(Forrow & Maltby, 2000)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
								$T = exposure\ time\ (days)$ $C_L = correction\ factor\ of\ autogenic\ changes\ in\ leaf\ weight$ $C_L = \frac{\Sigma\left(\frac{Lc_a}{Lc_b}\right)}{N}$ $Lc_a = control\ leaf\ discs\ final\ dry\ weight\ (mg)$ $Lc_b = control\ leaf\ discs\ initial\ dry\ weight\ (mg)$ $N = number\ of\ control\ replicates$	
Alnus glutinosa		Natural drilled groundwater		2.0 cm	21 days	**Leaves**: Photo Scanned at the beginning and after 7 days when replaced		$FR = \frac{\Sigma_{i=1}^{N} \frac{(S_t - S_{t-1})}{\frac{n_t + n_{t-1}}{2}}}{T}$ $FR = feeding\ rate$ $T = total\ experiment\ duration\ (days)$ $S_t = total\ surface\ of\ leaf\ discs\ in\ each\ container\ (mm^2)$ $n_t = number\ of\ living\ gammarids$	(Geffard et al., 2010)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
Alnus glutinosa	Antibiotics (added in the water during the conditioning process)	Pure natural water	Total Darkness	2.0 cm	3 hours	**Leaves**: **Before & After**: Photo Scanned **Organisms**: **After**: lyophilized for 18 h and stored in an exsiccator.		Leaf area was calculated by counting the pixel numbers of each single leaf disc.	(Hahn & Schulz, 2007)
Alnus glutinosa		*In situ*: river water		(Naylor et al., 1989)	6 days	**Leaves**: **Before**: (Naylor et al., 1989) **After**: 60˚C for 4 days **Organisms**: **After**: 60˚C for 4 days		$FR = \frac{(L_1 * C_L) - L_2}{W * 6}$ $FR = feeding\ rate$ $L_1 = initial\ leaves\ dry\ weight\ (mg)$ $L_2 = final\ leaves\ dry\ weight\ (mg)$ $W = organisms\ dry\ weight\ (mg)$ $C_L = leaf\ weight\ change\ correction\ factor$	(Maltby et al., 2002)
Alnus glutinosa	Fungicides	SAM-5S	Darkness	1.6-2.0 cm	24h 24 days	**Leaves**: **Before:** **After**: 60°C for 24h		Calculated following the method of (Zubrod et al., 2015)	(Newton et al., 2018)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
						Organisms: **After**: 60°C for 24h			
Alnus glutinosa	Anti-cholinesterase compounds			2.0 cm 5 for each tested concentration. Each beaker containing 20 organisms.		**Leaves**: **Before**: Photo Scanned and every 24 h		$FR_t = \frac{\sum_{i=1}^{5}\sum_{t=1}^{D}\left[\frac{S_{i,t}-S_{i,t-1}}{\frac{l_{i,t}+l_{i,t-1}}{2}}\right]/5}{D}$ $FR_t = Feeding\ rate$ $i = (i = 1-5)\ is\ the\ ith\ replicate$ $D =$ $is\ the\ Dth\ day\ during\ the\ experiment\ period$ $S =$ $total\ surface\ of\ leaf\ discs\ in\ each\ beaker\ (mm^2$ $l = number\ of\ living\ gammarids$	(Xuereb et al., 2009)
Alnus glutinosa	Fungicides tebuconazole	River water		Prepared following the method of (Bundschuh et al., 2011b).	7 days	**Leaves**: **Before:** **After**: 60°C **Organisms**:		$C = \frac{L_b * k - L_e}{g * t}$ $C = feeding\ rate$ $L_b = initial\ dry\ mass\ of\ the\ leaf\ discs\ (mg)$	(Zubrod et al., 2010)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
				2 leaf discs at the time		**After**: 60°C		$L_e = final\ dry\ mass\ of\ the\ leaf\ discs\ (mg)$ $g = dry\ weight\ of\ Gammarus\ fossarum\ (mg)$ $t = feeding\ time\ (days)$ $k = leaf\ change\ correction\ factor$ $k = \frac{\Sigma((L_{ob} - L_{oe})/L_{ob})}{n}$ $L_{ob} = initial\ dry\ mass\ of\ the\ leaf\ discs\ (mg)$ $L_{oe} = final\ dry\ mass\ of\ the\ leaf\ discs\ (mg)$ $n = number\ of\ replicates$	
Alnus glutinosa	Fungicides	SAM-5S		3.0 cm 2 leaf discs at the time	7 days	**Leaves**: **Before:** 60°C for 24 h **After**: 60°C for 24h **Organisms**: **After**: 60°C for 24 h		Calculate as described in (Zubrod et al., 2010)	(Zubrod et al., 2014)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
Alnus glutinosa	Fungicides	Bioassay medium		2.0 cm	6 days	**Leaves**: **Before:** **After**: 60°C for 24h **Organisms**: **After**: wet weight		$C = \frac{L_n - L_f}{t}$ $C = consumption\ of\ leaf\ material$ $L_n = dry\ weight\ of\ leaf\ discs\ prevented\ from\ feeding\ (mg)$ $L_f = dry\ weight\ of\ leaf\ discs\ available\ for\ feeding\ (mg)$ $t = exposure\ time\ (days)$	(Zubrod et al., 2015)
Populus sp.	Cadmium	Dutch standard water	Total darkness	Half a disc 3.0 cm in diameter	2 days	**Leaves**: **Before conditioning**: 60˚C for 48 h **After conditioning**: 60˚C for 48 h **After experiment**: 60˚C for 48 h **Organisms**: **After**: 60˚C for 48 h	4 days	$FA = \frac{F_i - F_f}{G}$ $FA = feeding\ activity$ $F_i = initial\ leaf\ dry\ weight\ (mg)$ $F_f = final\ leaf\ dry\ weight\ (mg)$ $G = dry\ weight\ of\ Gammarus\ (mg)$	(Alonso et al., 2009)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
Populus tremuloides		River water	12:12	Whole leaves 48 leaves each	3 days	**Leaves**: **Before**: 45 ˚C **After**: 45 ˚C **Organisms**: **After**: 45 ˚C		$RCR = \frac{\left[\frac{mg\ food\ ingested}{d}\right]}{mg\ mean\ animal\ weight} * 100 =$ $\%\ body\ weight\ weight\ consumed/day$ $RCR = relative\ consumption\ rate$	(Arsuffi & Suberkropp, 1989)
Quercus petraea	Microplastics	ISO medium		4 cm 1 disc every 8 days	48 days	**Leaves**: **Before:** 40°C **After**: 40°C **Organisms**: **After**: wet weight		Relative feeding rate calculate as mg leaf material consumed per mg body mass per day.	(Weber et al., 2018)
Ulmus procera		Artificial pond water		1.6 cm	1 day	**Leaves**: **Before**: 60°C for 4 days **After**: 60˚C for 4 days **Organisms**:		$FR = \frac{W_i - W_t}{t}$ $FR = feeding\ rate$ $W_i = mean\ weight\ of\ control\ discs\ (mg)$ $W_t =$ $final\ weight\ of\ leaf\ discs\ offered\ as\ food\ (mg$	(Graça et al., 1993a)

Leaf species	Contaminant	Water used for the experiment	Light: Dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying Temperature and Time (before and after exposure)	Starvation	Equation used	Reference.
						After: 60˚C for 4 days		$t = feeding\ period\ (days)$	

2.6 Conclusions

Feeding behaviour has been used to investigate the sublethal effects of a wide range of contaminants over the years. As well as providing information on an organism level, feeding studies could also be adapted to understand the possible effects on entire populations, and therefore potential threats to a population could be transposed, to understand the prospective repercussions on the ecosystem.

Throughout this review, it is noticeable that there are variations within the adopted methodologies for the acclimation conditions, the leaf conditioning process and the leaf species used. This review has also highlighted that several different equations are used in the literature to quantify the feeding rate of Gammarids.

During the acclimation period, the organisms are kept at temperatures ranging from 10 to 22°C, even though all of the species considered in this review are from temperate countries. Temperature has been proven to have a significant impact on Gammarids by affecting their physiological parameters and their immune system. Temperature could ultimately have an impact on their feeding rate, which increases when the temperature is raised. The authors recommend that a constant temperature is maintained during the acclimation period and the experiment itself, in order to have a reliable estimation of the feeding rate, independent of a temperature difference. Moreover, the acclimation and experimental temperature should reflect the average conditions for the country where the experiment is being undertaken. In fact, both Maltby et al. (2002) and Coulaud et al. (2011) demonstrated that temperature has a major impact on feeding rate variability during *in situ* experiments. Consequently, when an *in situ* experiment includes several different deployments in different geographical areas, temperature should be measured in each location, so that the impact of temperature on the feeding rate can be estimated. Furthermore, the media in which the organisms are acclimated should always be aerated.

Similarly, the authors recommend that light:dark cycles aiming to reproduce seasonal conditions should be avoided, in order to allow the reproducibility of a study regardless of the time of the year. However, this is not the case for *in situ* studies. The temperature and light:dark cycles during acclimation for *in situ* experiments should best replicate the natural environment. Consequently, natural

light:dark conditions and the air and water temperature should be measured, reported and replicated.

Ex situ experiments should be standardised (e.g. using an artificial medium if possible), meaning that the medium's parameters (i.e. pH, conductivity, total hardness) should be measured and reported, and the medium should be screened beforehand for contamination. If contamination is present, it is important to record the concentrations of the specific contaminant to identify the background concentration level, to have a better understanding of the possible effects. This is especially noteworthy when river water is used to acclimate the organisms, in particular for *in situ* experiments where river water mimics natural environmental conditions for the acclimation period. It is difficult to say how long the acclimation should last. The authors recommend that further research is required to determine the impact of time frame on acclimation periods and to determine if a longer acclimation period results in stronger data with a lower level of variability.

Several different food types have been highlighted during this review, including different leaf species and conditioning methods. Even though Gammarids are biologically omnivorous organisms, a leaf-based diet is recommended in feeding studies, both during the acclimation period and the exposure, and the same food should be used for both (i.e. same leaf species and same preparation). *Alnus* spp. are the most commonly used leaf material for freshwater Gammarid feeding studies. The authors therefore recommend *Alnus* spp. as a standardised food source for ecotoxicological assays. However, the distribution of *Alnus* spp. is not ubiquitous around the world, and therefore it might be challenging for some researchers to source them for their experiments. In such situations, industrial feed might be a better solution to overcome the problem of non-standardisation. If leaf material is used, applying a conditioning process is recommended, since conditioned material has been proven to be more palatable and have a lower C:N, which translates to a better energy supply. Moreover, it has also been demonstrated how leaf palatability increases when they are conditioned for longer time periods. Consequently, a short conditioning period (i.e. a few days) should be avoided, and organisms should be fed on leaves conditioned for at least 10 days. However, this time period should be prolonged if using fresh leaf material, since it has been reported that conditioning takes longer. In *ex situ*

experiments, conditioning should be conducted using an artificial media inoculated with *Cladosporium* spp., which is the most common fungi species used in the literature to condition leaves. This will ultimately reduce the likelihood of contamination that might result from using river detritus as a source of fungi inoculum for conditioning leaves.

On the other hand, for *in situ* studies, the conditioning process should ultimately replicate, as accurately as possible, real-life environmental conditions and processes, which means using river water, inoculum and Gammarids from the study site. As previously mentioned, the composition of the water needs to be identified and also the chemical parameters; the latter could then be replicated during the conditioning process. For *in situ* experiments, the authors recommend conditioning the leaf material directly in the river. For example, placing leaves in small net bags that are submerged and secured in the river where the experiment would take place. This would provide the Gammarids with the same type of food during the acclimation period and exposure. However, conditioning takes time, so it should be undertaken well in advance of the experiment.

It is still unclear if conditioning should take place before or after the leaves are cut into discs, dried in the oven and weighed. Consequently, the authors recommend that further investigations need to be undertaken to compare if drying the leaf discs in the oven should be undertaken before or after the conditioning process and if either of these methodologies alter the feeding rate of Gammarids. Organisms are usually fed *ad libitum* during the acclimation period. To further reduce the inner variability and strengthen the data, the authors recommend incorporating a starvation period in the experimental design. This starvation period should take place before the feeding experiment, and its purpose is to synchronise the organisms' hunger levels. The authors also recommend that organisms of a comparable size range should be used in experiments as it has been proven that Gammarids of different sizes have a different feeding rate. Juveniles are more sensitive to contaminants, but their feeding rate is characterised by a higher variability over time, which makes them more suitable for short-term studies. On the other hand, because of their greater sensitivity, juveniles are better for ecotoxicological studies by providing ecologically relevant risk assessments for contaminants. *Gammarus* spp. have been widely adopted for ecotoxicological studies, but the genus contains many different species, and

even though very similar, there are still differences in their sensitivities, meaning that the choice of one species over the other should be carefully considered, depending on the contaminant tested.

This is of particular interest for *in situ* experiments since the adopted species would be dependent on the site, but also dependent on the season, which could determine the availability of particular organism sizes. So in order to further reduce inner variability and allow better estimation of the feeding rate, organisms should be measured at the start of an experiment, possibly by photography and length measurements, in order to have a pool of organisms of the same size and potentially the same life stage. This is particularly noteworthy when growth is measured alongside the organisms feeding rate.

The source of the organisms might also have an impact on the results. Organisms collected in the wild could be better suited for *in situ* studies, as they could provide a more realistic site-specific response. However, local site-related species may be characterised by previous exposure histories that could ultimately influence their feeding rate (e.g. they could potentially be acclimated to a certain level of pollution). This is a problem that has to be taken into consideration both for *in situ* and *ex situ* experiments. Perhaps laboratory-bred organisms should be used to reduce variability even further, and it would provide a constant stock of Gammarids (Blockwell et al., 1996; Bloor & Banks, 2006a, b; McCahon & Pascoe, 1988). However, breeding Gammarids is not always possible and it is highly species dependent. Long-term culturing could also potentially lead to a higher or lower contaminant sensitivity and a reduced genetic variability.

The last step of a feeding study involves the quantification of the feeding rate by using an equation. As highlighted in this review (Table 2.3), there are various equations in the literature that are indiscriminately used to calculate the feeding rate. However, some of these equations are mathematically different, and it raises the question, are the equations and the feeding rates generated by them equal?

The feeding rate can be estimated by using the leaf weight or surface area. The equation that is most commonly adopted estimates the feeding rate by comparing leaf dry weight before and after being provided to the Gammarids, divided by the time (expressed in days) and the weight of the organisms. Usually the dry weight

of the leaf discs is adjusted with a constant. The authors recommend that the position of the constant is dependent on when the leaves are conditioned and dried. If the leaves are conditioned after being dried and weighed, the constant should multiply with the initial dry weight, so that it takes into consideration that the leaf disc might have lost more weight through being submerged in water during the experiment. However, if the leaf discs are dried and weighed after being conditioned, the constant should divide the final dry weight, because some of the leaf material might have been lost through leaching and not through Gammarid feeding.

Another consideration is that the constant is not always calculated in the same way, and this could ultimately alter the experimental results. Again, the authors recommend that further research is required to understand the impact of the various constant positions on the outcome of a study. Until then, the authors recommend that the equation provided by Maltby et al. (2002) is adopted, as it is representative of real-life environmental feeding.

When leaf area is used to calculate the feeding rate, the constant is not often included in the equation. This means that the possible loss of leaf material due to the leaching process is not taken into consideration. Leaf area is often used to calculate the feeding rate for *in situ* experiments, so the authors recommend that if this method is going to be used, a set of control leaves should also be established, in order to calculate a leaching constant[5] based on the difference in surface area.

It is clear that a standardised protocol is required, which would benefit the scientific community and regulatory authorities and allow them to interpret and compare published literature to understand the impact of various contaminants (and mixtures) on the environment. This could be achieved by undertaking serial experiments to clarify what impact these heterogeneities have on the final results. There are methodologies such as Naylor et al. (1989) and Nilsson (1974) that have been used many times, but unfortunately, there are still others that are the result of a mixed methodology. The variability within feeding studies has already

[5] From Chapter 3 onwards, the leaching constant will be referred to as Leaf Change Correction Factor or simply Correction Factor (CF).

been acknowledged, and the first steps towards standardisation have evolved (Agatz & Brown, 2014).

A standardised *ex situ* methodology would greatly benefit this field of research, by not only allowing a more meaningful comparison between the peer-reviewed literature, but also to better understand the impact that specific contaminants could have on Gammarid populations and ecosystems. This could be enhanced further if *ex situ* experiments are placed side by side with biomarker analysis and *in situ* studies. In theory, *in situ* tests could provide a realistic and integrated understanding of real environmental pollution. If standardised, *in situ* tests could be used by regulators to critically evaluate the state of an ecosystem and the potential impact that a certain contaminant or mixture could have on the environment. This is of particular interest since the establishment of the Water Framework Directive (European Union 2000), which outlines that all European water bodies should reach 'good quality status' by 2015 and has since been extended. The establishment of a standardised suite of *in situ* and *ex situ* feeding assays would provide a realistic monitoring tool and environmental risk assessment, which would be of benefit to the scientific community, and also decision makers.

Chapter 3: Different amphipod feeding rate calculation methods lead to varying conclusions: a case study using the antidiabetic drug, metformin.

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Author contribution:

All the data presented in this chapter were collected, processed and interpreted by Consolandi, G. Statistical analyses were carried out by Consolandi, G. with the help and supervision of Parker, M.O. Consolandi, G. drafted the manuscript. All authors contributed and revised the final manuscript.

3.1 Abstract

Gammarus pulex (*G. pulex*) is common freshwater detritivore that is often used as a test species in ecotoxicology to study the effects of different classes of contaminants. *G. pulex* plays a key role in the decomposition and breakdown of organic matter in lotic environments. For this reason, its feeding behaviour is often adopted as a sublethal endpoint and it can be calculated mathematically. In the literature there are several different equations that can be used to calculate *G. pulex* feeding activity. Therefore, the aim of this study was three-fold: 1) to determine if different feeding equations lead to the same conclusions, 2) to determine if two different methodologies (one based on the weight of leaves and one based on the leaf disc surface area) give the same results and 3) to understand if the antidiabetic metformin (MET), one of the world's most prescribed pharmaceuticals, has an impact on *G. pulex* feeding activity and swimming velocity. Two experiments were set up (24 h and 7 day) and organisms were exposed to three concentrations of MET (0.1 μg/L, 1 μg/L and 10 μg/L). When the feeding rate was calculated as leaf area consumed, two different equations (Equation 1 and 2) were applied and compared, whereas for the consumed lead mass three different equations were used (Equation 3, 4 and 5). The results showed that Equation 1 and 2, used to calculate the leaf area consumed, could be considered equivalent. Whereas, Equation 5, of the three equations adopted to calculate the consumed leaf mass, produced results that

did not reflect the real feeding activity of *G. pulex*. The two methodologies (leaf area consumed and consumed leaf mass) were not equally sensitive in the 7 days experiment. MET did not have an effect on the feeding activity of *G. pulex* after 24 h. In the 7-day experiment, the feeding rate was estimated after 2 and 5 days (when leaf discs were replaced) and again at the end, after 7 days. A 10 μg/L MET concentration was found to inhibit *G. pulex* feeding activity (calculated as consumed leaf mass) after 2 days, compared to the control (p=0.013). MET did not have an effect on the swimming velocity of *G. pulex*. The present study demonstrates that different methodologies (leaf area consumed and consumed leaf mass) can be used to calculate the feeding activity of *G. pulex* and that the consumed leaf mass might be more sensitive in longterm exposures. It was also demonstrated that Equation 5 produces unrealistic results and should therefore not be adopted. Finally, MET can have adverse effects on the freshwater amphipod *G. pulex* at 10 μg/L.

Keywords: Behavioural studies, Feeding equations, Feeding rate, *Gammarus pulex*, Metformin, Pharmaceuticals

3.2 Introduction

Ecotoxicologists are increasingly using behavioural endpoints to understand the sub-lethal impact that different classes of contaminants can have on organisms in the natural environment (Klamider et al., 2016). One such endpoint is the feeding activity of the detritivore (shredder) *Gammarus pulex (G. pulex*) [Linnaeus], a water column dwelling amphipod that is sensitive to a wide range of contaminants (Adam et al., 2009; Bloor et al., 2005; Kunz et al., 2010; McCahon & Pascoe, 1988c; Vellinger et al., 2012).

The breakdown of coarse particulate organic matter (CPOM) is brought about by a combination of microbial decomposition, macroinvertebrate feeding, chemical leaching, and physical abrasion. Previous studies have shown that conditioning of leaf material by fungi increases its palatability to macroinvertebrate shredders and that aquatic hyphomycetes, in particular, play an important role in the

microbial decomposition of leaf material (Nelson, 2011). The processing of CPOM by microorganisms and shredders produces fine particulate organic matter (FPOM), which is consumed by filter feeders and collector-gatherers. The latter are in turn consumed by invertebrate and vertebrate predators (Cummins, 1974). Hence, efficient decomposition is key to the energy budget (and therefore the integrity) of many stream ecosystems (Maltby, 1994). For this reason, change in the feeding rate (FR) of *G. pulex* is often used as a sub-lethal endpoint to investigate the effects of specific pollutants.

Even though feeding behavioural studies with Gammarids have been carried out for over half a century, there is still not a universal standardised methodology and there are many variations in the protocol (Consolandi et al., 2019). Consolandi et al. (2019) identified several areas where standardisation within feeding studies should be adopted by the ecotoxicology community to allow comparisons between the published literature, assist with policy makers and regulation. One such area is the standardisation of the equations used to calculate the FR, since the use of several different equations is published in the literature. Two techniques commonly used for estimating the FR are: 1) measuring the difference in surface area consumed by the organisms (Hahn & Schultz, 2007), 2) measuring the difference in weight of the food, before and after the experiment (Maltby et al., 2002).

One of the main differences is the way that the FR is calculated; in other words, the equations that are used to quantify the effective feeding activity are different. These equations essentially estimate the same endpoint, but the factors position can vary and so can the way that they are calculated. This is particularly true for the Leaf Change Correction Factor (CF)[6] also known as the leaching constant. The CF is a constant that takes into account the loss of leaf material in water due to natural decomposition processes. The CF can be placed in different positions within the equation or it can be estimated using different formulae (Agatz et al., 2014; Bundschuh et al., 2011b; Bundschuh et al., 2017; Maltby et al., 2002).

Patients with diabetes have a high level of sugar in their blood because they cannot produce insulin (Type 1 diabetes) or they cannot use it (Type 2 diabetes).

[6] In Chapter 2, the Leaf Change Correction Factor is referred to as constant or leaching constant.

Hundreds of millions of people suffer from diabetes worldwide (Scheurer et al., 2012; IDF Diabetes Atlas, 2017) and 90% of those people, have Type 2 diabetes (WHO, 2015). Metformin (MET) is one of the most commonly prescribed medications to treat type 2 diabetes (Briones et al., 2016). MET is very effective, convenient and it has recently been adopted as primary active ingredient in several combinational antidiabetic drugs (Trautwein et al., 2014). The demand for MET is expected to increase further since it has been identified as a new possible treatment for other illnesses (Kasznicki et al., 2014; Martin-Castillo et al., 2010; Niemuth & Klaper, 2015). In the last decade, MET has also been adopted as a treatment to help weight loss (Seifarth et al., 2013), as it has been suggested that MET promotes weight loss by regulating appetite pathways in the brain (Malin & Kashyap, 2014). MET is not metabolised when ingested by the human body and up to 70% of the therapeutic dose is excreted in its original form in urine and feces (Gong et al., 2012). The prescribed daily dose of MET is usually of 2 g (WHO Collaborating Centre for Drug Statistics Methodology, 2019), consequently it is perpetually discharged into the environment at very high concentrations.

MET, along with many new-generation molecules, is not completely removed by Wastewater Treatment Plants (WWTPs) and its removal efficiency has been reported to vary from 40 to 98% (Blair et al., 2015; Scheurer et al., 2012; Briones et al., 2016). Across Europe, varying concentrations of MET have been reported in freshwater environments. Samples from several different Greek WWTPS influents and effluents were examined and MET concentrations up to 1167 ng/L (influents) and 26 ng/L (effluents) were reported (Kosma et al., 2015). In the Netherlands, the MET influent concentration was 79 µg/L compared to 1.5 µg/L in the effluent (Oosterhuis et al., 2013). While Scheurer et al. (2009) reported a median concentration of 110 µg/L and 11.4 µg/L, respectively, when sampling influent and effluent in Germany. In Lake Constance, the MET concentration was found to be between 35 and 150 ng/L and even higher concentrations were detected in the river Elbe (472 ng/L) and the river Weser (349 ng/L), (Trautwein et al., 2014). France recorded MET concentrations as high as 735 ng/L (Vulliet & Cren-Olivé, 2011) and in the UK, an annual median concentration up to 1117 ng/L was measured in the River Foss (Burns et al., 2018).

Outside Europe, MET was detected in Malaysia with a concentration up to 293 ng/L in the River Langat and up to 16 ng/L in effluent water (Al-Odaini et al., 2010). In South Africa, MET was measured with a mean concentration of 73.3 ng/L upstream of a WWTP and 174.6 ng/L downstream of the plant (Archer et al., 2017), and Lake Michigan (North America) recorded a median value of 100 ng/L (Blair et al., 2013a).

Pharmaceuticals are active ingredients designed to work at low concentrations, consequently they might induce effects in non-target organisms with similar metabolic pathways, receptors or biomolecules (Ankley et al., 2007; Santos et al., 2010) and they may affect functions such as behaviour, growth, reproduction and development (Ebele et al., 2017). MET induces a reduction of the blood glucose level by inhibiting complex I of the electron transport chain, which will lead to a decrease in ATP levels and the activation of the enzyme AMP kinase (AMPK). This improves the glucose uptake into cells, as well as inhibiting hepatic gluconeogenesis and glycogenolysis (Viollet et al., 2012).

From an aquatic ecotoxicological prospective, research with MET has been undertaken with different fish species (Jacob et al., 2018; Niemuth & Klaper, 2015; Ussery et al., 2018), freshwater gastropods (Jacob et al., 2019), brachiopoda (Caldwell et al., 2019) and rotifers (García-García et al., 2017). To the authors knowledge, the present study is the first to look at the effects of MET on *G. pulex* and to undertake a comparison of the different equations used to determine FR.

The aim of this study is three-fold: 1) to examine the implications of using different equations to estimate the FR of *G. pulex* when exposed to the antidiabetic drug MET, and to identify if these equations generate equivalent results for standardisation purposes; 2) to determine the comparability between leaf area consumed or consumed leaf mass; and 3) to identify if MET affects the feeding activity and swimming velocity of *G. pulex*. It is hypothesised that the *G. pulex* FR may be inhibited by the presence of MET and subsequently, changes in the organism's feeding activity could potentially influence the organism's energy budget, and therefore have an impact on their swimming velocity.

3.3 Material and methods

3.3.1 Chemicals

Metformin hydrochloride (CAS Number: 115-70-4) was purchased from Sigma-Aldrich (United Kingdom).

3.3.2 Preparation of the leaf discs

Freshly abscised but undecomposed black alder (*Alnus glutinosa*) leaves were handpicked in October 2016 from a single tree in Sir Harold Hillier Gardens, Romsey, UK (51˚00'47.3''N; 1˚27'53.8''W). The leaves were taken back to the laboratory at the University of Portsmouth, air dried and stored at room temperature in the dark until use. Dried leaves were soaked in charcoal filtered tap water for 2 h and subsequently, 1.3 Ø cm discs were cut from each leaf with a plunger cutter, avoiding the main veins. Leaf discs were then dried in an oven at 60˚C for 24 h and weighed to the nearest of 0.1 mg. Two weeks prior to the start of each experiment, individual leaf discs were conditioned in river water that was collected along with the organisms from the River Ems, Westbourne, UK (50˚51'40.3''N; 0˚55'42.9''W). After the two-week conditioning process, the leaves were individually rinsed in bottled water (Evian®) (Table 3.1), photographed with a Leica MC120 HD camera mounted on a stereo microscope (Leica S8APO) and provided to the organisms for the feeding experiment (Appendix C).

3.3.3 Test organisms

River water and *G. pulex* specimens were collected two weeks prior the start of each feeding experiment from the River Ems, Westbourne, UK (50˚51'40.3''N; 0˚55'42.9''W) using a hand-net. Parameters (total hardness, nitrate, total alkalinity and phosphate) (Table 3.2) of the river water were determined with colourimetric test kits (CHEMets® and HACH®). Conductivity, pH and oxygen saturation were also measured (Table 3.2). Organisms infected with the acanthocephalan parasite (*Pomphorhynchus laevis*) were discarded, since this parasite has been proven to affect the feeding activity of its host (Pascoe et al., 1995). *G. pulex* were taken to the University of Portsmouth to

acclimate the organisms to laboratory conditions. Adult males (mean dry weight = 5.71 ± 1.57 mg (Experiment 1) and 6.07 ± 1.57 mg (Experiment 2)) were isolated and kept at 15±0.1˚C under a 12:12 light:dark cycle for two weeks in a 3 L aquarium filled with bottled water (Evian®; pH=7.2) (Table 3.1) and fed *ad libitum* with *Alnus glutinosa* leaves. Prior to the start of the experiment, the leaves were naturally conditioned in aerated river water for at least two weeks. After the two-week acclimation phase, the organisms were starved for 48 h before the start of each experiment, in order to ensure a standardised hunger state.

Bottled water (Evian®) was used with the purpose of standardisation. Evian® is natural mineral water and its mineral composition was in line (e.g. hardness) with the river water parameters from our designated collection site (Table 3.1 and 3.2).

Table 3.1. Evian® mineral composition in mg/L.

Mineral composition (mg/L)	
Sodium Na^+	6.5
Silicia SiO_2	15
Bicarbonates HCO_3^-	360
Sulphates SO_4^{2-}	14
Nitrates NO_3^-	10
Calcium Ca^{2+}	80
Magnesium Mg^{2+}	26
Potassium K^+	1
Dry residue 180°C	345 mg/L
pH	7.2

Table 3.2. River water parameters.

	pH	Temp.(˚C)	Total Hardness (as $CaCO_3$) (mg/L)	Nitrate (mg/L)		Total Alkalinity (mg/L)	Conductivity (uS)	Phosphate (mg/L)	Dissolved O_2 (mg/L)
				30 sec	60 sec				
24 h experiment	7.2	13.5	425	0	10	240	587	<1	~ 8
7-day experiment	8.0	16.2	425	0	5	240	550	<1	~ 8.8

3.3.4 Feeding experiments

Two separate feeding experiments were set up to investigate the possible effects of the antidiabetic drug MET on *G. pulex*. The first experiment investigated changes in the FR over 24 h, whereas the second experiment investigated changes over 7 days. In both experiments, three different environmentally realistic concentrations of MET were tested. Each treatment included 15 replicates, consisting of one specimen of *G. pulex* in a polypropylene pot filled with 100 mL of bottled water (Evian®; pH=7.2) (for the experimental controls) or 100 mL of bottled water with a nominal concentration of either 0.1 μg/L, 1 μg/L or 10 μg/L of MET. For the 24 h experiment, each *G. pulex* was provided with one leaf disc at 15°C, in the dark. After the 24 h feeding time and the behavioural analyses (described below), the *G. pulex* were sacrificed by freezing at -20°C and the leaf discs were photographed (Appendix C). The *G. pulex* specimens and the leaf discs were then dried in a GenlabPrime oven (Genlab Ltd, UK) for 24 h at 60°C and subsequently weighed to the nearest 0.1 mg.

The 7-day experiment was undertaken in the dark at 15°C with each organism being provided with two leaf discs that were replaced at day 2 and day 5 when water changes were undertaken. Once removed from the test chamber, each leaf disc was photographed again and dried at 60°C for 24 h and weighed to the nearest of 0.1 mg. At the completion of the 7 day experiment (FR and behavioural analyses), the *G. pulex* specimens were sacrificed by freezing at -20°C and dried in the oven for 24 h at 60°C and weighed to the nearest of 0.1 mg.

For each experiment, 15 control leaf discs were also established, in order to calculate area and/ or mass loss during the conditioning process and the

experiment itself. These control leaf discs underwent the same process as the other leaves used in the experiment, but they were not fed to the organisms.

3.3.5 Behavioural analyses

After the 24 h (Experiment 1 and 2) and 7-day (Experiment 2) experiments, the *G. pulex* swimming velocity was recorded using a 6-well plate (Kohler et al., 2018) in a Noldus DanioVision™ observation chamber connected to a Noldus EthoVision® XT 11.5 video tracking software (Tracksys, Nottingham, UK). The observation chamber was equipped with an infrared sensitive camera and a holder for a multiwell plate. Additionally, the holder could be backlit with a cold white light that could be programmed. After the 24 h experiments (Experiment 1 and 2), each organism was gently transferred with the test medium from their experimental pot into one of the wells of the 6-well plate. Once the organisms were in place, the 6-well plate was placed in the Noldus DanioVision™ observation chamber (Figure 3.1), where organisms were left to acclimate to the new test conditions for one minute. The velocity (cm/s) of each specimen was recorded for 6 minutes under a 3-minute dark: 3-minute light cycle with a 50% light intensity (2000 lx). A 3-minute dark: 3-minute light cycle was chosen in order to investigate the behavioural response of *G. pulex* to a disturbance (i.e. light) (Kohler et al., 2018). After this time, the organisms were either sacrificed (Experiment 1) or transferred back into their experimental pots for the remainder of the experiment (Experiment 2). In the 7-day experiment, each organism underwent the same process for a second time on the final day of the experiment, before being sacrificed.

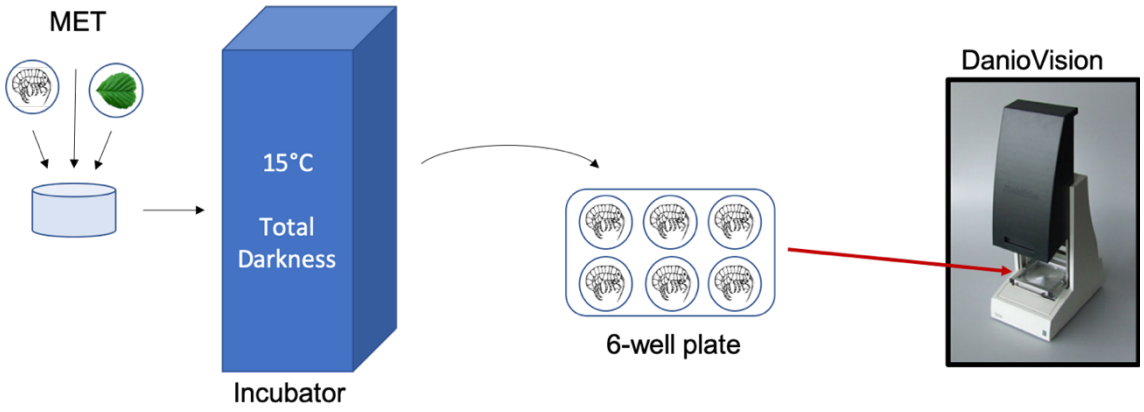


Figure 3.1. Schematic explanation of the experimental set-up using the DanioVision™ observation chamber.

3.3.6 Data analyses

G. pulex feeding rate (FR) was measured either as leaf area consumed (Equations 1 and 2) or as consumed leaf mass (Equations 3, 4 and 5).

Equations 1 and 2 differ by the inclusion of a leaf change correction factor (CF_A) in Equation 2, calculated using changes in the control leaf area (Equation a):

(1) $FR = \frac{A_i * (CF_A) - A_f}{w * t}$ (a) $CF_A = \frac{\left[\Sigma\left(\frac{A_{cf}}{A_{ci}}\right)\right]}{n}$

(2) $FR = \frac{A_i - A_f}{w * t}$ (Hahn & Schulz, 2007),

where A_i is the initial area of the leaf disc (mm^2), A_f is the final area of the leaf disc (mm^2), w is the animal dry weight (mg), t is the feeding time (days). CF_A is the leaf change correction factor (Equation a), where A_{ci} is the initial area of the control leaf discs (mm^2), A_{cf} is the final area of the control leaf discs (mm^2) and n is the number of replicates.

Equations 3, 4 and 5 express the feeding rate (FR) as consumed leaf mass per mg of dry mass of the organism and time. Each equation also accounts for the leaf change correction factor (CF); however, this constant is either in a different position in the equation (Equation 3 and 4) or is calculated in a different way (Equation b and c):

(3) $FR = \frac{L_i - \frac{L_f}{CF_1}}{w * t}$ (Agatz & Brown, 2014)

(4) $FR = \frac{L_i * (CF_1) - L_f}{w * t}$ (Maltby et al., 2002)

(5) $FR = \frac{L_i * (CF_2) - L_f}{w * t}$ (Bundschuh et al., 2011b)

where L_i is the initial dry weight of the leaf disc (mg), L_f is the final dry weight of the leaf disc (mg), w is the animal dry weight (mg), t is the feeding time (days) and CF is the leaf change correction factor. In the context of Equation 3-5 CF was calculated with the following equations:

(b) $CF_1 = \frac{\left[\sum\left(\frac{C_f}{C_i}\right)\right]}{n}$

(c) $CF_2 = \frac{\sum\left[\frac{C_i - C_f}{C_i}\right]}{n}$,

where C_i is the initial dry weight of the control leaf discs (mg), C_f is the final dry weight of the control leaf discs (mg) and n is the number of replicates.

The data were analysed using IBM SPSS (version 24). Normality was first verified and then the significant area or mass loss was established either by Univariate Analysis of Variance (Experiment 1) or by a Linear Mixed Effects Model (Experiment 2), (type error rate for all models: α =0.05) for all the different equations and comparisons, with exposure time (days) and concentration set as fixed factors and organisms' ID as random effect. A Linear Mixed Effects Model was also applied to estimate changes in the *G. pulex* swimming velocity (Experiment 1 and 2), with time (as seconds spent in the dark or in the light in the DanioVision™ observation chamber), concentration and exposure (days; Experiment 2) set as fixed factors and organisms' ID as random effect. A Bonferroni adjustment was applied to the α level for all pairwise or post-hoc comparisons and possible interactions (Experiment 2).

3.4 Results

3.4.1 Experiment 1

3.4.1.1 Feeding behaviour

Regardless of the equation used (see Equation 1 and 2), there were no significant changes in the amount of leaf area consumed by *G. pulex* when exposed to MET for 24 h (Equation 1: Univariate Analysis of Variance: F(3,51)=0.246 *p*=0.864, Figure 2), (Equation 2: Univariate Analysis of Variance: F(3,51)=0.248 *p*=0.862, Figure 2) (Table 3.3). The area loss due to natural decomposition was calculated through the leaf change correction factor CF_A (equation a) and $CF_A = 0.97882$. Consequently, when CF_A was multiplied by the initial surface area A_i, it did not have a great impact on the results obtained from Equation 1 and Equation 2, that differed because of the constant (Figure 3.2).

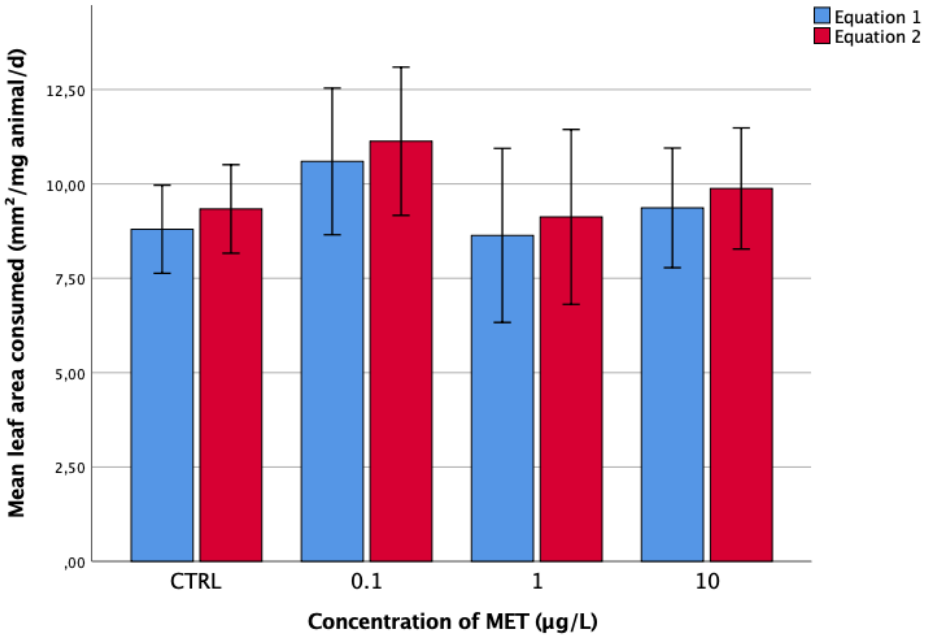


Figure 3.2. Mean leaf area consumed (± standard error) when calculated with Equation 1 and 2 by *G. pulex* when exposed to different concentrations of the antidiabetic drug MET over a period of 24h.

Similarly, no significant difference was measured in the consumed leaf mass by *G. pulex* (Figure 3.3) (Table 3.3). When Equation 3 and 4 were used the

Univariate Analysis of Variance was applied (F(3,51)=0.390, *p*=0.761) (Figure 3.3). By using Equation 5 the data became mainly negative, meaning that the organisms didn't eat (Univariate Analysis of Variance: F(3,51)=0.649, *p*=0.587) (Figure 3.3).

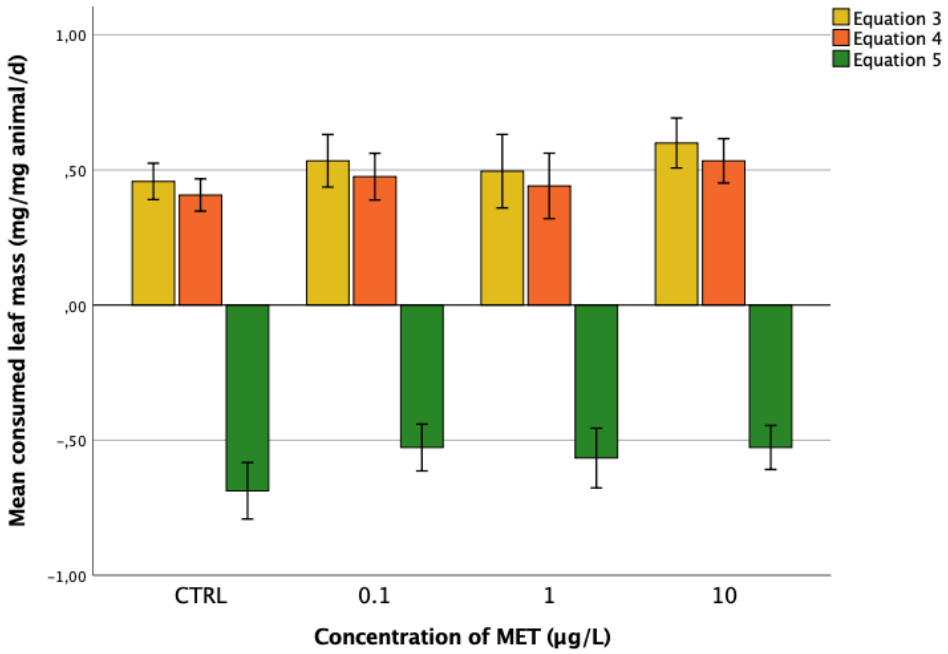


Figure 3.3. Mean consumed leaf mass (± standard error) when calculated with Equation 3, 4 and 5 by *G. pulex* when exposed to different concentrations of the antidiabetic drug MET over a period of 24h.

Table 3.3. Univariate Analysis of Variance of *G. pulex* feeding rate (n=55) after 24 h exposure to MET.

		Source	df	Mean square	F	*p*
Dependent variable: leaf area (mm²/mg animal/d)	**Equation 1**	Concentration	3	10.436	0.246	0.864
		error	51			
	Equation 2	Concentration	3	10.688	0.248	0.862
		error	51			
Dependent variable: leaf weight (mg/mg animal/d)	**Equation 3**	Concentration	3	0.053	0.390	.761
		error	51			
	Equation 4	Concentration	3	0.042	0.390	0.761
		error	51			
	Equation 5	Concentration	3	0.084	0.649	0.587
		error	51			

3.4.1.2 Swimming velocity

After 24 h exposure, *G*. *pulex* swimming velocity (Figure 3.4) was not significantly different between the treatments (F(3,56)= 2.544 ; p=0.065) and they reacted similarly when the light was turned on across the different concentrations (F(105,1960)=0.672; p=0.995). Organisms swam significantly faster when the light was on compared to when it was off (F(35,1960)=28.632; p<0.001), but this behaviour was unrelated to the concentration of MET they were exposed to (Table 3.4).

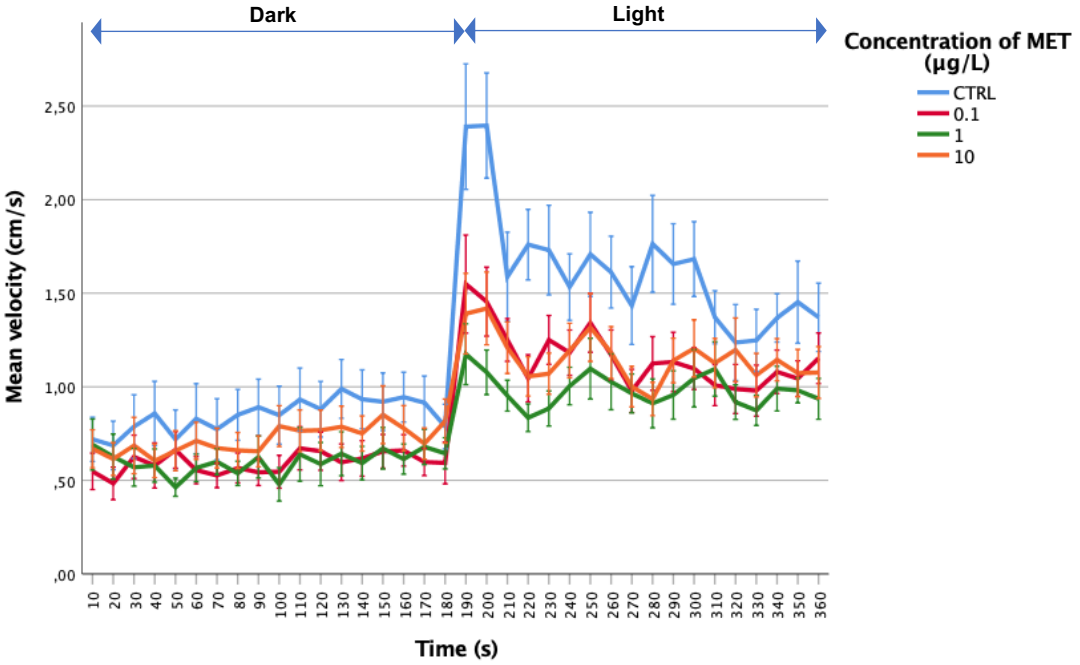


Figure 3.4. Mean velocity of *G. pulex* when exposed to different concentrations of the antidiabetic drug MET for 24 h. Error bars indicate standard error (± Standard Error).

Table 3.4. Linear Mixed Effect Model of *G. pulex* velocity (n=60) after 24 h exposure to MET. Concentration is to indicate the different concentration tested and time indicates the time spent inside the DanioVision™ chamber, namely 3 minutes dark: 3 minutes light photoperiod.

Dependent variable: swimming velocity (cm/s)				
Source	**Numerator df**	**Denominator df**	**F**	***p***
Concentration	3	56	2.544	0.065
Time	35	1960	28.632	<0.001
Concentration*time	105	1960	0.672	.995

3.4.2 Experiment 2

3.4.2.1 Feeding behaviour

In the second experiment, *G. pulex* feeding rate was measured over a period of 7 days while being exposed to MET (Table 3.5). When Equation 1 and 2 were applied to the data the leaf area consumed showed a decreasing trend,

which was consistent within the different concentrations (Figure 3.5A and 3.5B). No significant difference was observed between the different concentrations (Linear Mixed Effect Model: $F(3,49.3)=0.619$, $p=0.606$; $F(3,49.3)=0.623$, $p=0.604$) with Equation 1 and 2 respectively (Figure 3.5A and 3.5B). Similarly, regardless of the Equation (1 and 2), there was no interaction between concentration and time (Linear Mixed Effect Model: $F(6,97.7)=0.683$, $p=0.664$; $F(6,97.7)=0.685$, $p=0.662$), meaning that the organisms ate a similar amount of leaf area throughout the length of the experiment. However, an overall significant decrease in leaf area consumption was detected, when Equation 1 (Linear Mixed Effect Model: $F(2,97.7)=16.966$, $p<0.001$) and Equation 2 (Linear Mixed Effect Model: $F(2,97.7)=16.752$, $p<0.001$) were used. Pairwise comparisons (Table 3.6) showed that the amount of leaf area eaten after 2 days was significantly different from the quantity consumed at day 5 (Equation 1, mean difference (2>5) = 2.33 ± 0.49, $p<0.001$; Equation 2, mean difference (2>5) = 2.23 ± 0.49, $p<0.001$) and day 7 (Equation 1, mean difference (2>7) = 2.6 ± 0.49, $p<0.001$; Equation 2, mean difference (2>7) = 2.6 ± 0.49, $p<0.001$).

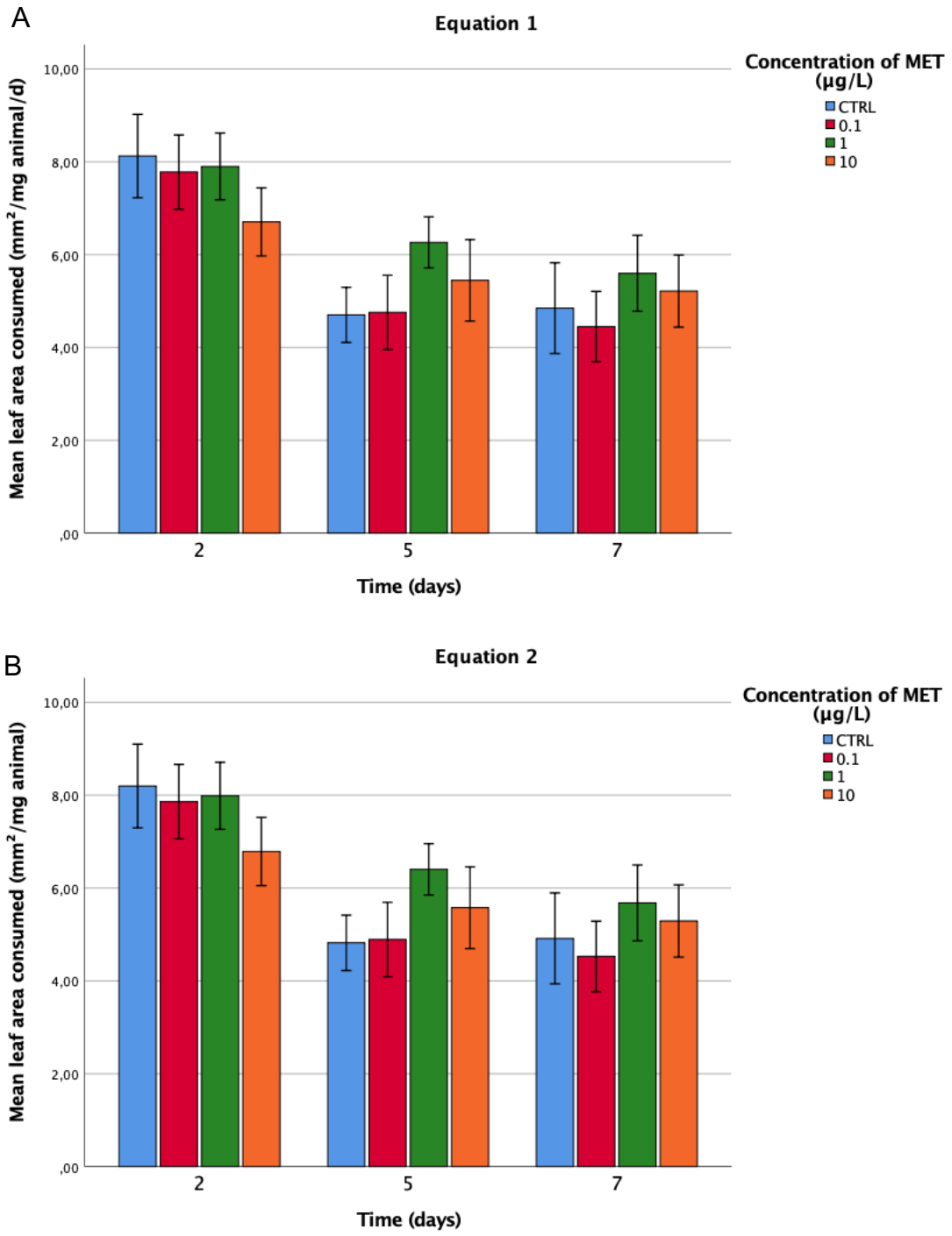


Figure 3.5. Mean Leaf area consumed (± standard error) by *G. pulex* when exposed to different concentrations of the antidiabetic drug MET for 7 days. (A) No significant difference was measured between the concentrations (Linear mixed effect model: F(3, 49.3)=0.619, p=0.606) and no significant interaction between concentration and exposure time (Linear mixed effect model: F(6, 97.7)=0.683, p=0.664). A significant overall decrease in leaf consumption was detected over time (Linear mixed effect model: F(2, 97.7)=16.966, p<0.001). (B) No significant difference was detected between the concentrations (Linear mixed effect model: F(3, 49.3)=0.623, p=0.604) and no significant interaction between concentration and exposure time (Linear mixed effect model: F(6, 97.7)=0.685, p=0.662). There was an overall significant decrease in leaf area consumption over time (Linear mixed effect model: F(2, 97.7)=16.752, p<0.001).

Table 3.5. Linear Mixed Effect Model of *G. pulex* feeding rate (n=53) over an exposure period of 7 days to MET.

Dependent variable		Source	Numerator df	Denominator df	F	p
leaf area (mm²/mg animal/d)	Equation 1	Concentration	3	49.295	0.619	0.606
		Exposure	2	97.728	16.966	<0.001
		Concentration*Exposure	6	97.715	0.683	0.664
	Equation 2	Concentration	3	49.294	0.623	0.604
		Exposure	2	97.725	16.752	<0.001
		Concentration*Exposure	6	97.712	0.685	0.662
	Equation 3	Concentration	3	49	1.302	0.284
		Exposure	2	98	9.304	<0.001
		Concentration*Exposure	6	98	3.000	0.010
leaf weight (mg/mg animal/d)	Equation 4	Concentration	3	49	1.049	0.379
		Exposure	2	98	3.940	.023
		Concentration*Exposure	6	98	2.622	0.021
	Equation 5	Concentration	3	49	1.359	0.266
		Exposure	2	98	136.986	<0.001
		Concentration*Exposure	6	98	2.036	0.068

Table 3.6. Pairwise comparisons for *G. pulex* feeding rate at different exposure times (2 days, 5 days and 7 days).

Dependent variable: leaf area (mm²/mg animal/d)						
	Exposure (I)	**Exposure (J)**	**Mean difference (I-J)**	**Std. Error**	**df**	***p***
Equation 1	2 days	5 days	2.333	0.493	97.903	<0.001
		7 days	2.599	0.490	97.385	<0.001
	5 days	2 days	-2.333	0.493	97.903	<0.001
		7 days	0.266	0.493	97.903	1.000
	7 days	2 days	-2.599	0.490	97.385	<0.001
		5 days	-0.266	0.493	97.903	1.000
Equation 2	2 days	5 days	2.285	0.493	97.899	<0.001
		7 days	2.606	0.490	97.383	<0.001
	5 days	2 days	-2.285	0.493	97.899	<0.001
		7 days	0.320	0.493	97.899	1.000
	7 days	2 days	-2.606	0.490	97.383	<0.001
		5 days	-320	0.493	97.899	1.000

In comparison to the 24 h experiment, in the 7-day experiment the consumed leaf mass showed a similar trend compared to the leaf area consumed, in particular

after 2 days for organisms exposed to the higher concentration (10 µg/L) (Figure 3.6). After 2 days the mean leaf area consumed was not significantly different compared to the control or to the other two tested concentrations of 0.1 µg/L and 1 µg/L (Equation 1, 6.705 ± 0.757; Equation 2, 6.787 ± 0.759), whereas the mean consumed leaf mass (Equation 3, 0.202 ±0.047; Equation 4, 0.147 ± 0.38) was found to be conflicting with the results obtained with Equation 1 and 2 as a significant difference was measured (Figure 3.6).

The results obtained from Equation 3 and 4 (Figure 3.6A and 3.6B) were different from the results obtained with Equation 5 (Figure 3.7) (Table 3.5).

When the data were calculated with Equation 3 and 4, no significant difference was observed between the concentrations (Equation 3, Linear Mixed Effect Model: $F(3,49)=1.302$, $p=0.284$; Equation 4, Linear Mixed Effect Model: $F(3,49)=1.049$, $p=0.379$). However, the amphipods consumed a significantly lower amount of leaf over time (Equation 3, Linear Mixed Effect Model: $F(2, 98)=9.304$, $p<0.001$; Equation 4, Linear Mixed Effect Model: $F(2,98)=3.940$, $p=0.023$) and a significant interaction between concentration and exposure time was detected (Equation 3, Linear Mixed Effect Model: $F(6,98)=3.000$, $p=0.010$; Equation 4, Linear Mixed Effect Model: $F(6,98)=2.622$, $p=0.021$), which means that the organisms consumed a different amount of leaf across the concentrations after 2 days, 5 days and 7 days (Equation 3, mean difference (2>5) = 0.086 ± 0.32, $p=0.028$; mean difference (2>7) = 0.137 ± 0.32, $p<0.001$; Equation 4, mean difference (2>7) = 0.07 ± 0.026, $p=0.025$), (Table 3.5 and 3.7).

In particular after 2 days, organisms exposed to the highest concentration of MET (10 µg/L) ate a significant smaller amount of leaf compared to the control (Equation 3, mean difference (control>10 µg/L) = 0.257 ± 0.079, $p=0.013$; Equation 4, mean difference (control>10 µg/L) = 0.187 ± 0.058, $p=0.013$) and to the median concentration (1 µg/L) (Equation 3, mean difference (1 µg/L >10 µg/L) = 0.230 ± 0.076, $p=0.024$; Equation 4, mean difference (1 µg/L >10 µg/L) = 0.168 ± 0.055, $p=0.024$) (Table 3.8).

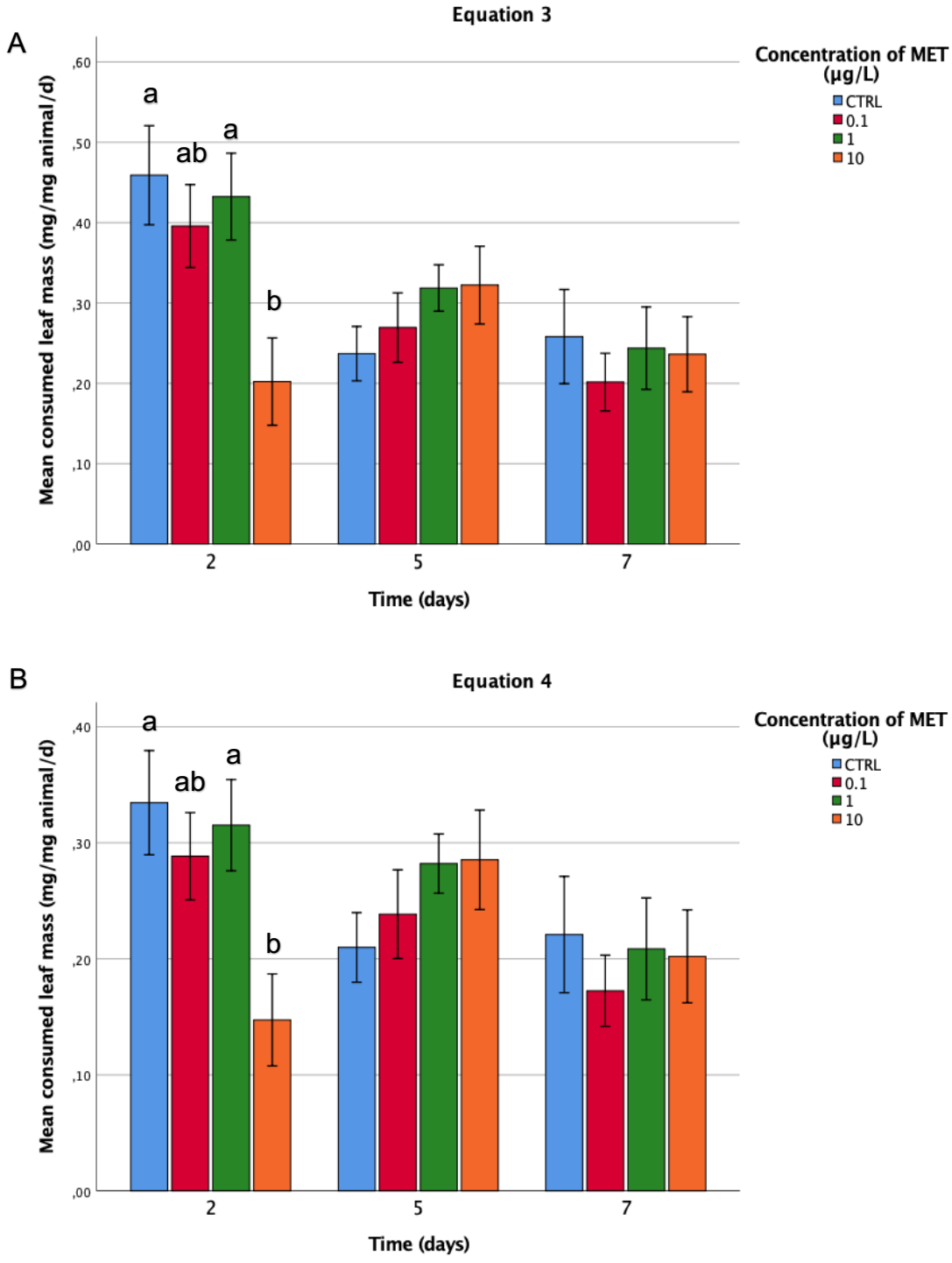


Figure 3.6. Mean consumed leaf mass (± standard error) by *G. pulex* when exposed to different concentrations of the antidiabetic drug MET for 7 days. Letters indicate significant differences between different concentrations within the same exposure time.(A) Consumed leaf mass calculated with Equation 3 and (B) Equation 4.

When Equation 5 was applied (Figure 3.7) (Table 3.5), the amphipods consumed a statistical different amount of leaf over the duration of the experiment, regardless of the concentration they were exposed to (Linear Mixed Effect Model: $F(2,98)=136.986$, $p<0.001$) (Table 3.7). However, no statistical difference was detected within the different concentrations (Linear Mixed Effect Model: $F(3,49)=1.359$, $p=0.266$) and no interaction within concentration and exposure time (Linear Mixed Effect Model: $F(6,98)=2.036$, $p=0.068$).

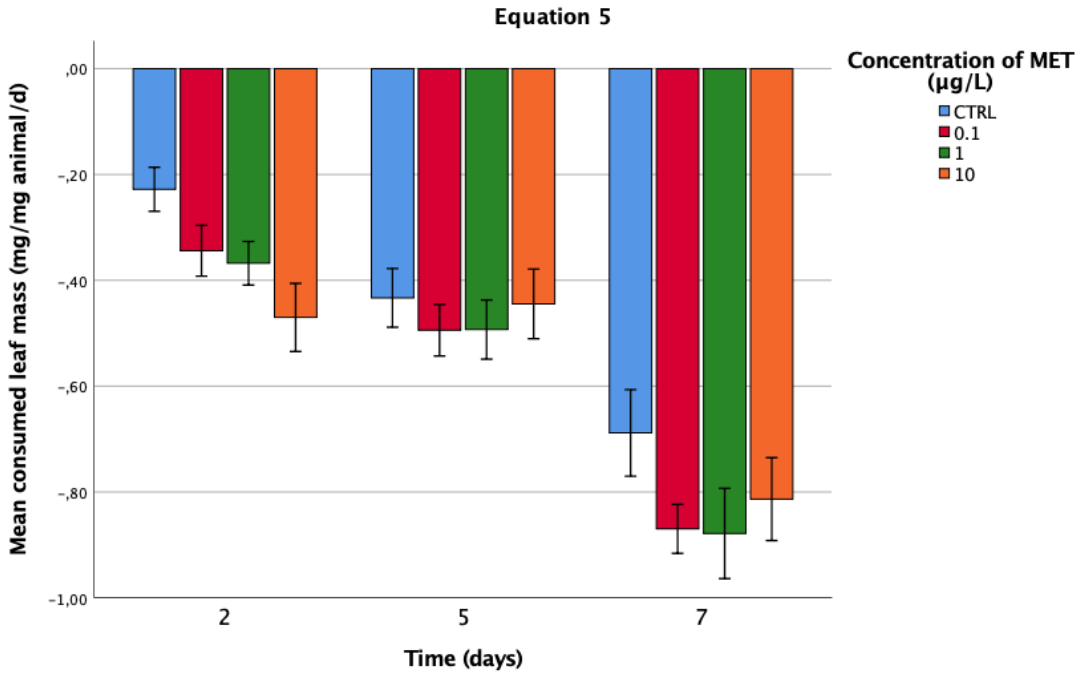


Figure 3.7. Mean consumed leaf mass by *G. pulex* when exposed to different concentrations of the antidiabetic drug MET for 7 days: No significant difference was detected between the concentrations (Linear mixed effect model: $F(3, 49)=1.359$, $p=0.266$) and no significant interaction between concentration and exposure time (Linear mixed effect model: $F(6, 98)=2.036$, $p=0.068$). There was an overall significant decrease in leaf area consumption over time (Linear mixed effect model: $F(2, 98)=136.986$, $p<0.001$).

Table 3.7. Pairwise comparisons for *G. pulex* feeding rate measured at different exposure times (2 days, 5 days and 7 days).

Dependent variable: leaf weight (mg/mg animal/d)						
	Exposure (I)	**Exposure (J)**	**Mean difference (I-J)**	**Std. Error**	**df**	***p***
Equation 3	2 days	5 days	0.086	0.032	98	0.028
		7 days	0.137	0.032	98	<0.001
	5 days	2 days	-0.086	0.032	98	0.028
		7 days	0.052	0.032	98	0.330
	7 days	2 days	-0.137	0.032	98	<0.001
		5 days	-0.52	0.032	98	0.330
Equation 4	2 days	5 days	0.017	0.026	98	1.000
		7 days	0.070	0.026	98	0.025
	5 days	2 days	-0.17	0.026	98	1.000
		7 days	0.053	0.026	98	0.136
	7 days	2 days	-0.070	0.026	98	0.25
		5 days	-0.053	0.026	98	0.136
Equation 5	2 days	5 days	0.008	0.002	98	<0.001
		7 days	0.033	0.002	98	<0.001
	5 days	2 days	-0.008	0.002	98	<0.001
		7 days	0.025	0.002	98	<0.001
	7 days	2 days	-0.033	0.002	98	<0.001
		5 days	-0.025	0.002	98	<0.001

Table 3.8. Pairwise comparisons of the feeding rate of *G. pulex* after 2 days exposure. Concentrations are expressed in µg/L

	Exposure: 2 days					
	Dependent variable: leaf weight (mg/mg animal/d)					
	Concentration (I)	**Concentration (J)**	**Mean difference (I-J)**	**Std. Error**	**df**	***p***
Equation 3	CTRL	0.1	0.063	0.081	49	1.000
		1	0.027	0.079	49	1.000
		10	0.257	0.079	49	0.013
	0.1	CTRL	-0.063	0.081	49	1.000
		1	-0.037	0.077	49	1.000
		10	0.194	0.077	49	0.096
	1	CTRL	-0.027	0.079	49	1.000
		0.1	0.037	0.077	49	1.000
		10	0.230	0.076	49	0.024
	10	CTRL	-0.257	0.079	49	0.013
		0.1	-0.194	0.077	49	0.096
		1	-0.230	0.076	49	0.024
Equation 4	CTRL	0.1	0.046	0.059	49	1.000
		1	0.019	0.058	49	1.000
		10	0.187	0.058	49	0.013
	0.1	CTRL	-0.046	0.059	49	1.000
		1	-0.027	0.056	49	1.000
		10	0.141	0.056	49	0.096
	1	CTRL	-0.019	0.058	49	1.000
		0.1	0.027	0.056	49	1.000
		10	0.168	0.055	49	0.024
	10	CTRL	-0.187	0.058	49	0.013
		0.1	-0.141	0.056	49	0.96
		1	-0.168	0.055	49	0.024

3.4.2.2 Swimming velocity

In the second experiment *G. pulex* swimming velocity was measured initially after 24 h (Figure 3.8A) and again after 7 days (Figure 3.8B) of MET exposure. Similarly to the 24 h experiment, no significant difference was measured in the swimming velocity between the treatments ($F(3,56)=2.486$, $p=0.070$) (Table 3.9), and the organisms significantly increased their velocity when the light was turned on after 3 minutes in the dark ($F(35,3976)=50.302$, $p<0.001$) (Table 3.9). There was no interaction between treatment and time ($F(105,3976)=0.941$; $p=0.652$), meaning that their increased velocity when exposed to light was not related to the treatment they were exposed to during the experiment. The organisms' velocity was significantly different (slower in the dark) after 7 days compared to after 24 h ($F(1,3976)=400.171$; $p<0.001$) but also in this case, there was no interaction with the treatments ($F(3,3976)=0.362$; $p=0.780$). A significant interaction between time and exposure was observed ($F(35,3976)=10.945$; $p<0.001$), suggesting that organisms swam faster when the light was switched on after 7 days compared to after 24 h (Figure 3.8A and 3.8B). Finally there was no three-way interaction (concentration*time*exposure), indicating that MET did not have an effect on how *G. pulex* responded to light after 24 h and after 7 days ($F(105,3976)=0.560$; $p=1.000$).

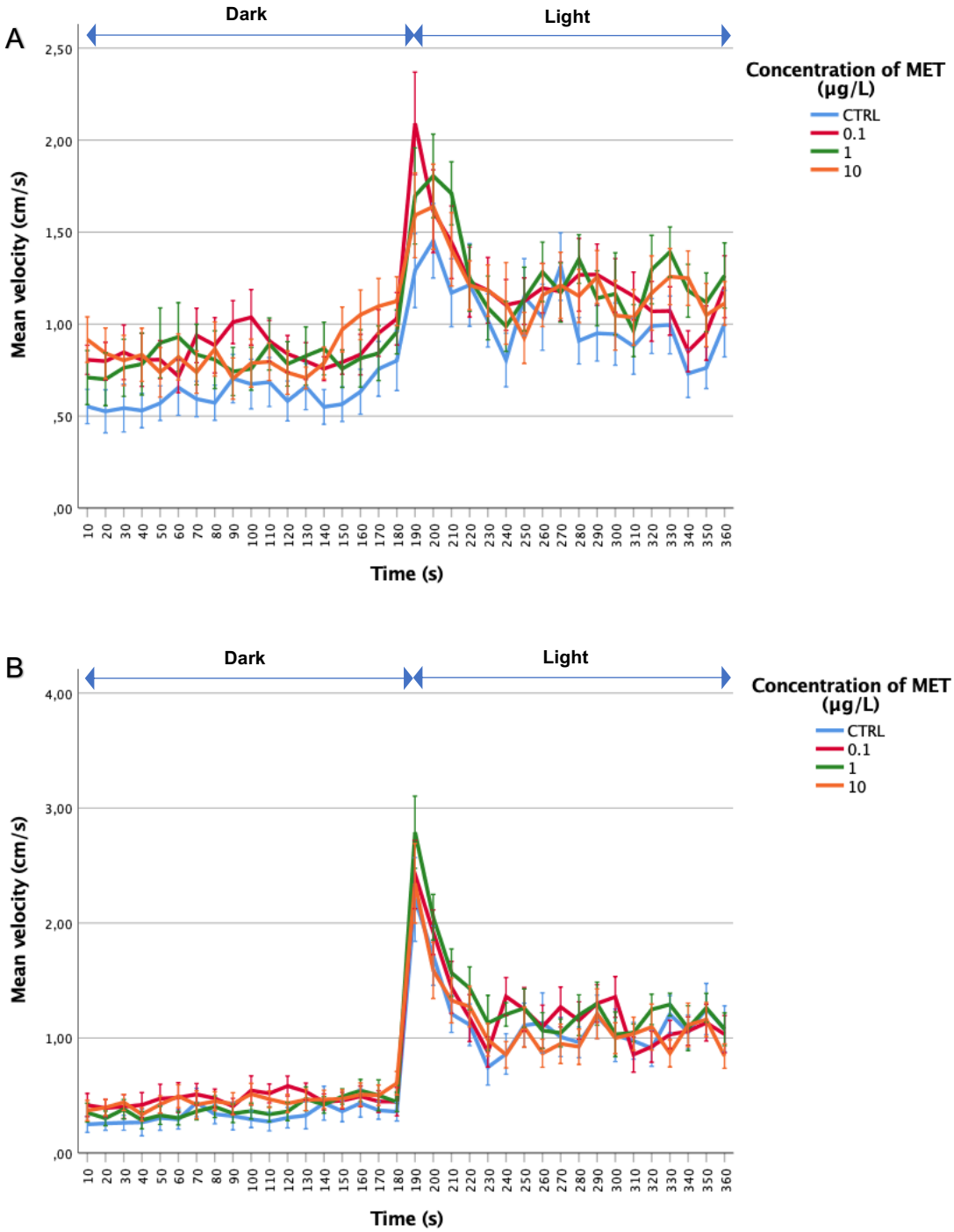


Figure 3.8. Mean velocity of *G. pulex* when exposed to different concentration of the antidiabetic drug MET for (A) 24h and (B) 7 days. Error bars indicate standard error (± Standard Error).

Table 3.9. Linear Mixed Effect Model of *G. pulex* velocity (n=60) after exposure to MET, measured after 24 h and 7 days. Concentration is to indicate the different concentration tested (0.1, 1 and 10 µg/L). Time indicates the time spent inside the DanioVision™ chamber, namely 3 minutes dark: 3 minutes light. Exposure indicates after how much time organisms' velocity was measured in the DanioVision™ chamber (i.e. 24 h and 7 days).

Dependent variable: swimming velocity (cm/s)				
Source	**Numerator df**	**Denominator df**	**F**	***p***
Concentration	3	56	2.486	0.070
Time	35	3976	50.302	<0.001
Exposure	1	3976	400.171	<0.001
Concentration*Time	105	3976	0.941	0.652
Concentration *Exposure	3	3976	0.362	0.780
Time*Exposure	35	3976	10.945	<0.001
Concentration*Time*Exposure	105	3976	0.560	1.000

3.5 Discussion

The aim of the current study was three-fold: (1) to understand if the use of different equations to estimate the FR of *G. pulex* leads to the same conclusions, 2) to determine if the FR can be calculated either as leaf area consumed or consumed leaf mass, and 3) to identify if MET affects the FR and swimming velocity of *G. pulex*.

Our study demonstrates that the antidiabetic drug MET did not have an impact on the FR of *G. pulex* after a 24 h exposure, regardless of which equation was applied to the data. The use of photo analysis (Equation 1 and 2) to calculate the difference in surface area proved to be an accurate method of measurement and was comparable to the traditional method of using leaf weight (Equation 3 and 4). Even though there was no significant difference within the different concentrations, due to the different way of calculating the leaf change correction factor (Equation C), the data became negative when Equation 5 was used, suggesting a non-existent feeding activity. Photo analysis, which was based on

the pixel size, contradicts these results, confirming the results obtained from Equation 3 and 4 instead.

Regardless of which equation was applied to the data (for both methodologies, weight or surface area), MET did not have an impact on the FR of *G. pulex* after 24 h, which suggests that the use of photo analysis to calculate the difference in the surface area might be considered an accurate method of measurement, and can be comparable to the traditional method of using leaf weight. However, in the 7-day experiment the two methodologies used to quantify the FR generated different results. No significant difference was found in the leaf area consumed, whereas significant differences were identified when the consumed leaf mass was measured, in particular with Equation 3 and 4. Nevertheless, both methods showed a similar trend and the organisms exposed to the highest concentration (10 µg/L) had the lowest feeding activity. Collectively these results suggest that calculating the FR by identifying changes in leaf weight might generate more accurate data, especially in long-term experiments, because it is based on a multidimensional measure.

To our knowledge, this is the first study to apply both methods simultaneously to calculate the FR in order to understand the level of comparability. The quantification of the FR as leaf area consumed has been carried out for over a decade and it has been applied in *ex situ* experiments (Hahn & Schulz, 2007; Geffard et al., 2010; Xuereb et al., 2009) and more often in *in situ* experiments (Coulaud et al., 2011; Dedourge-Geffard et al., 2009). In *in situ* experiments, FR is usually calculated as the loss in leaf surface related to the number of Gammarids that are still alive. The authors believe that because *in situ* experiments usually do not have replicates of a single organism, this approach might not be feasible or practical as FR is usually calculated on several animals. Nonetheless, no CF is usually integrated into the formula. Coulaud et al. (2011) adjusted their equation by incorporating the surface of control leaves that were not fed to the Gammarids. However, these control leaves were not used to generate a constant. To the best of our knowledge, this is the first study to incorporate a CF based on surface area and the authors believe that Equation 1 provides the most accurate results. Especially for *in situ* experiments, where the natural river environment might have a greater impact on the decomposition and loss of material compared to the more stable setting of laboratory conditions.

Our study also demonstrates that the use of different equations to quantify FR can lead to different conclusions when calculated as consumed leaf mass. In both the 24 h and 7 day experiments, statistical analyses showed that Equation 3 and Equation 4 can be considered equivalent. Equation 3 and 4 differ because of the CF_1 position within the formula. Consolandi et al. (2019) highlighted this discrepancy and suggested that the position of the CF within the equation should depend on the method used to prepare the leaves. Indeed, it was suggested that the position of CF_1 is dependent on the method used to prepare the leaf discs for the feeding experiment.

Agatz et al. (2014) used Equation 3 in their study and the leaf discs were first conditioned, then dried and weighed before being supplied to the Gammarids. In this case, the authors believe that CF_1 compensates for a potential loss of material during the experiment itself, since the loss in material during the conditioning process has already been taken into account. On the other hand, Maltby et al. (2002) used Equation 4 in their research and in that case, the leaves were dried and weighed before undergoing the conditioning processes. Consequently, CF_1 compensates for any possible changes made to the initial weight by conditioning the leaves.

However, when Equation 5 was applied to the data, negative values were obtained that implied no feeding activity had taken place, which contradicted what was visually observed and the photo analysis itself. Moreover, statistical analyses confirmed that Equation 5 generated results that led to misleading conclusions, in particular for the 7-day experiment. Equation 5 differs from Equation 3 and 4 by how the CF is calculated. CF_1 appears to calculate for the amount of leaf that is left after the conditioning process, whereas the authors believe that CF_2 estimates for the amount of leaf material that is lost during conditioning. By using our data, the obtained CF_2 value was much smaller than CF_1 ($CF_2 << CF_1$), so when multiplied with the initial dry weight L_i, it resulted in $(L_i * CF_2) < L_f$ and ultimately, negative FR values. Consequently, the authors discourage the use of Equation 5 because the results generated are not representative of real feeding activity. Equation 5 has been used by different research studies such as, Bundschuh et al. (2011b) and Zubrod et al. (2010), and the authors of the current study believe that it should not be used to calculate the FR for Gammarids. The

authors also want to acknowledge that studies following on from the aforementioned examples have already corrected the equation (e.g. Bundschuh et al., 2017), and CF_2 has been re-written as ($1\text{-}CF_2$), confirming our hypothesis that CF_2 calculates the amount of leaf material lost during the conditioning.

Consequently, the authors believe that only data generated from Equation 3 and 4 can be considered representative of real *G. pulex* feeding activity. Therefore, the present study demonstrated that the FR of *G. pulex* is inhibited when exposed to 10 µg/L of MET. These results are in line with previous studies found in the literature. Jacob et al. (2019) reported a decrease in weight and a reduced feeding activity of the freshwater big Ramshorn snail (*Planorbarius corneus*) when exposed to 0.1 mg/L, 1 mg/L and 10 mg/L. As in the current study, they encountered a high variability within their samples and unfortunately were unable to fully quantify the effective food uptake.

On the other hand, the concentrations tested by Jacob et al. (2019) were relatively high, making comparison difficult. Nevertheless, a reduction in body weight was reported for different fish species that were exposed to environmentally relevant concentrations of MET. Jacob et al. (2018) reported that brown trout larvae (*Salmo trutta* f. *fario*) showed a significantly reduced body weight when exposed to a MET concentration range of 1-100 µg/L. In another study, Japanese medaka (*Oryzias latipes*) growth was significantly reduced after exposure to 1-100 µg/L MET for 28 days (Ussery et al., 2018). Furthermore, an environmentally relevant MET concentration of 40 µg/L was found to significantly decrease the weight of adult male fathead minnows (*Pimephelas promelas*), (Niemuth & Klaper, 2015). The reported weight loss on exposure to MET is in accordance with our initial hypothesis.

MET has recently been proposed as a medication for weight-loss in obese patients (Seifarth et al., 2013). Even though MET has been on the market (in Europe) for more than half a century (Bailey, 1992), it is only recently that scientists have started to unravel and explain its different modes of action. Among them, it has been proposed that MET might have an anorectic effect (Glueck et al., 2001; Kim et al., 2013). However, the exact mechanism by which MET regulates appetite is still unclear (Lv et al., 2012) and this is even more obscure for invertebrates. Nonetheless, assuming that MET might have a similar effect of

Gammarids, the results imply that the *G. pulex* 's FR was inhibited by 10 µg/L of MET after 2 days.

Several types of lipids, proteins and glycogen are the most important metabolic macromolecules in Crustacea (Jimenez & Kinsey, 2015). These metabolic fuels can be either synthetized or assimilated thought the diet. But most importantly, some of them can be oxidised to produce adenosine triphosphate (ATP), (Jimenez & Kinsey, 2015). Glucose in Crustacea is mainly absorbed through the diet and is usually stored as glycogen, in the hepatopancreas and muscles (Oliviera et al., 2003). When the level of glucose in the haemolymph falls below a certain threshold, the glycogen is broken down into glucose through the glycogenolysis pathway. However, one of MET's main modes of action is to inhibit glycogenolysis (Viollet et al., 2012). Therefore, *G. pulex* exposed to MET should theoretically have a lower glucose level, as feeding is inhibited, but also lower level of ATP, which is necessary for energy metabolism (Musin et a., 2017). Consequently, MET could have an effect on the swimming velocity of *G. pulex* as a reduction in feeding may translate into a lower energy intake, which not only could have an effect on movement and velocity but inevitably might have potential repercussions at a population level, by exposing organisms to higher risk of predation (De Lange et al., 2006).

In the present study, MET did not have an impact on the *G. pulex* swimming velocity. In both experiments, even though there was no significant difference in swimming velocity between the treatments, the test statistics (F-values) suggested that an effect was likely, but the significance level (*p*-values) shows that the statistics are under-powered to detect that effect. Jacob et al. (2018) investigated the effect of MET on the swimming behaviour of brown trout larvae and also found that there was no significant effect.

Moreover, the 24 h and 7 day experiments showed conflicting results and differences were also observed between the experimental controls. Behavioural ecotoxicology is an expanding study area that could potentially link chemical changes and the effects on a population, and at an ecosystem level (Sloman & Mcneil, 2012). However, behavioural analyses are still widely characterised by a high intra-variability that complicates the reproducibility of these tests and reduces their reliability.

The increasing number of studies that show relatively high concentrations of MET in aquatic ecosystems has prompted the scientific community to research and understand what the potential implications of MET are on the natural environment. To the best of the authors' knowledge, this is the first study to look at the possible effects of MET on Gammarids, and specifically on *G. pulex*. However, it is recommended that further analysis is needed to better understand both the behavioural effects and the biochemical repercussion that MET may cause.

3.6 Conclusions

The present study has demonstrated that the feeding rate of *G. pulex* can be estimated with two different methodologies (as leaf area consumed or as consumed leaf mass). However, the two methods were not equally sensitive for long-term exposures. It was also demonstrated that not all equations used to calculate the consumed leaf mass can be considered equivalent for Gammarid studies. In the present study, MET did not have an impact on the swimming velocity of *G. pulex*, but it did have an impact on the FR of *G. pulex* after 2 days at a concentration of 10 µg/L. The inhibition of the feeding activity might translate in a reduced physiological fitness, limited growth and impaired reproduction, which could ultimately have repercussions on the population size. In freshwater ecosystems, *Gammarus* spp. can be the dominant macroinvertebrate in terms of biomass. They are common prey for different species of fish, birds and amphibians and they play a central role in leaf litter breakdown. Consequently, an inhibition in *G. pulex* feeding activity could have profound consequences on the ecosystem functioning. A MET concentration of 10 µg/L is at least 10 times higher than the concentrations reported in natural freshwater environments, but it can be considered environmentally relevant in areas of effluent discharge (see Table 1.1). Future research is required to investigate the impact that similar relevant concentrations might have on more sensitive life stages (e.g. gammarids juveniles) and female organisms, in order to have a more complete understanding of MET effects on *G. pulex*.

Chapter 4: Effects of an antibiotic mixture (sulfamethoxazole and trimethoprim) on the feeding rate of the freshwater detritivore *Gammarus pulex*.

Giulia Consolandi, Matt O. Parker, Mirco Bundschuh and Alex T. Ford.

Author contribution:

All the data presented in this chapter were collected, processed and interpreted by Consolandi, G. Fungal biomass analysis were carried out by Bundschuh, M. Statistical analyses were carried out by Consolandi, G. with the help and supervision of Parker, M.O. Consolandi G, drafted the manuscripts. All authors contributed and revised the final manuscript.

4.1 Abstract

The presence of pharmaceutical compounds in the aquatic environment is nowadays a well-established issue, that has raised concern in both the scientific and public community. Antibiotics are one of the many classes of pharmaceuticals that can be detected in freshwater environments in the ng/L to μg/L range. Antibiotics can affect the microbial communities that, along with freshwater detritivores are involved in the decomposition of coarse particulate organic matter (CPOM) in lotic ecosystems. The aim of the present study was to investigate the impact that an antibiotic mixture comprising sulfamethoxazole (SMX) and trimethoprim (TMP) might have on the natural leaf conditioning process and subsequent feeding rate of the freshwater amphipod *Gammarus pulex* (*G. pulex*). Leaf discs were exposed for 14 days to different concentrations of SMX and TMP added in an equal amount (1:1), each with a nominal concentration of either 2 µg/L, 20 µg/L and 200 µg/L. Leaf disc were then offered to *G. pulex* specimens for 24 h. *G. pulex* were found to eat significantly less (p<0.05) compared to the control when they were provided with leaf discs that were conditioned in the 2 μg/L and 20 μg/L mixture. A similar trend was also observable for the 200 μg/L concentration, although it was not significant. The

fungal biomass (as ergosterol) on the antibiotic conditioned leaves was also measured, but no significant difference was found. In contrast, the bacterial abundance in water was significantly lower in the 20 μg/l and 200 μg/L concentrations. Overall these findings suggest that a mixture of SMX and TMP may indirectly affect the feeding activity of the freshwater amphipod *G. pulex* and the bacterial communities in the water.

Keywords: Antibiotics, Feeding rate, Freshwater, *Gammarus pulex*, Sulfamethoxazole, Trimethoprim

4.2 Introduction

As a civilisation, we have become increasingly dependent on chemicals over recent decades, the list of such products is endless but examples include: pesticides, fertilisers, cosmetics and pharmaceuticals (Altenburger et al., 2018; Wilkinson et al., 2017). Many of these substances do not completely degrade or metabolise in humans or in wastewater treatment plants (WWTPs) and are therefore, released into the aquatic environment (Tijani et al., 2016). Consequently, wastewater influents are characterised by the presence of complex chemical mixtures that fluctuate in concentration and composition. It is suggested that chemical mixtures are one of the main challenges facing our freshwater ecosystems and our understanding of their impact is limited (Kienzler et al., 2016). To date, water quality testing for mixtures of contaminants is rarely done but new methodologies and approaches are being developed in order to evaluate mixture toxicity (Backhaus & Faust, 2012; Carvalho et al., 2014).

Since the 1970s and the first discovery of pharmaceuticals in the environment, aquatic ecotoxicologists have focused on understanding the potential impact that these individual compounds could have on natural ecosystems (Bernhardt et al., 2017). We know for example that pharmaceuticals are often only partially metabolised and degraded by the human body and during wastewater treatment which causes challenges (Carvalho & Santos, 2016). The production and consumption of pharmaceuticals is constantly increasing and consequently, so is their discharge into the environment where they can actively interfere with human- and animal-alike pathways in non-target organisms (Santos et al., 2010).

Over the years, numerous studies have reported the presence of many different classes of pharmaceuticals (e.g. anti-inflammatory, antidepressants, blood lowering agents, antibiotics) with their respective concentrations in the environment (Gracia-Lor et al., 2011; Kasprzyk-Hordern et al., 2007; Seifrtová et al., 2008). Among them are antibiotics which are of particular concern, as their unintentional release has led to the development of antibiotic resistant pathogens and they can also directly affect natural microbial communities (Kümmerer, 2009b).

Antibiotics are compounds designed to combat infections and diseases caused by bacteria in both human and veterinary medicine (Kümmerer, 2009a). Their consumption has grown exponentially since their first discovery at the beginning of the 20th century (Gothwal & Shashidhar, 2014). Even though wastewater effluents are considered one of the main routes of contamination (Kasprzyk-Hordern et al., 2009; Schwarzenbach et al., 2006), antibiotics can also reach the aquatic environment through run-off after application of manure to agricultural fields (Knäbel et al., 2016) and as growth promoters or to reduce microbial infections in aquaculture (Binh et al., 2018; Rico et al., 2013).

Antibiotics present two major modes of action (MOA), which are killing bacteria (bactericidal) or inhibiting their growth (bacteriostatic), so it is not surprising that when they are released into natural ecosystems, they have also been found to affect the natural microbial communities (Johansson et al., 2014). In freshwater environments, bacteria and fungi play an important role in the decomposition of organic matter, both directly and indirectly. Microbes can directly promote enzymatic organic matter degradation and indirectly by making it more palatable to shredders by conditioning the surface (Gessner et al., 1999). The enhanced palatability promotes macroinvertebrate detritivores to fragmentate and physically break colonised organic matter.

One such macroinvertebrate is the freshwater amphipod *Gammarus pulex* (*G. pulex*), [Linnaeus]. *G. pulex* is a widely distributed species in the northern Hemisphere, in particular in Europe and the British Isles (Karaman & Pinkster, 1977a; Kunz et al., 2010; Väinölä et al., 2008), and because of its broad distribution, *G. pulex* has often been used as a test species in ecotoxicological

studies (Kunz et al., 2010). Additionally, *G. pulex*, along with other amphipods, is sensitive to a variety of contaminants, both organic and inorganic (Wogram & Liess, 2001). *G. pulex* is a leaf-shredding detritivore and usually feeds on natural organic material, such as leaf litter that has previously been colonised by a layer of microbes (Bärlocher, 1985). Fungi and bacteria, by colonising the surface of organic material, increase its palatability and energy content. This process facilitates the transformation of terrestrial coarse particulate organic matter (CPOM) to fine particulate organic matter (FPOM), and consequently introduces it to the freshwater environment (Cummins & Klug, 1979). For this reason, *G. pulex* feeding behaviour, as well as other Gammarids, is often used to assess the potential impact of different contaminants (Blockwell et al., 1998; De Castro-Catalá et al., 2017; Lahive et al., 2015). This is because changes to *G. pulex* feeding activity could possibly impact the entire ecosystem by affecting the re-introduction of organic material in to the river flow from the terrestrial environment. Moreover, *G. pulex* is also known to be a common prey for fish, birds and amphibians (MacNeil et al., 2000).

The effects of chemicals are often studied individually using standardised ecotoxicological tests that ignore their combined effects (Altenburger et al., 2018). However, an increased awareness of this discrepancy is reflected by the number of studies that are now considering the combined effects of contaminants of anthropogenic origin and the debate on risk assessment of chemical mixtures (Backhaus & Karlsson, 2014). This study focuses on a mixture of two antibiotics, sulfamethoxazole (SMX) and trimethoprim (TMP). SMX and TMP are two antibiotics that are commonly prescribed together in human medicine to treat different conditions, such as respiratory, gastrointestinal and urinary infections (Ahmed et al., 2017; Cai & Hu, 2017). Additionally, they are often used in livestock farming and aquaculture as growth promoters (Binh et al., 2018; De Liguoro et al., 2012; Gothwal & Shashidhar, 2015; Paula et al., 2008). SMX and TMP have both been detected in surface waters in concentrations from ng/L to μg/L. In Australia, Watkinson et al. (2009) reported environmental concentrations of SMX and TMP as high as 2 μg/L and 0.15 μg/L, respectively. SMX was measured at a concentration of 0.3 μg/L downstream of a WWTP in a north American river

(Brown et al., 2006). In south Africa, Archer et al. (2017) reported average downstream concentration of SMX and TMP of 1,01 μg/L and 0.89 μg/L.

Therefore, the aim of this study is to understand if the antibiotic mixture of SMX and TMP has an impact on the bacterial communities in water and/ or the natural leaf conditioning process, and consequently whether they influence the feeding rate of the freshwater amphipod *G. pulex*. Previous studies have already addressed the effects of individual antibiotics and some mixtures on Gammarids' feeding (Bundschuh et al., 2009, 2017; Maul et al., 2006). However, SMX and TMP are currently prescribed together and to date, they have not undergone investigation as a mixture or with *G. pulex*, which makes this research extremely relevant.

4.3 Material and methods

4.3.1 Test organisms

River water and *G. pulex* specimens were collected two weeks' prior the start of the feeding experiment from the River Ems, Westbourne, UK (50˚51'40.3''N; 0˚55'42.9''W) using a hand-net. The location was chosen as there are no WWTPs discharging into the river north of the collection point. Parameters of the river water (total hardness, nitrate, total alkalinity and phosphate) were measured by using colourimetric test kits (CHEMets® and HACH®). Conductivity, pH and oxygen were also estimated with a portable conductivity tester (Hanna® Instrument Ltd, UK) and with a hand-held dissolved oxygen meter (OxyGuard® International A/S, Denmark), (Table 4.1). Organisms showing signs of infection by the acanthocephalan parasite *Pomphorhynchus laevis* were discarded as this parasite has been proven to affect the feeding behaviour of its host (Pascoe et al., 1995).

G. pulex were taken back to a laboratory at the University of Portsmouth where they were transferred in a 3 L aquarium filled with bottled water (Evian®; pH=7.2), kept at 15±0.1˚C under a 12:12 light:dark cycle for two weeks. During this time, *G. pulex* were fed *ad libitum* with alder leaves (*Alnus glutinosa*) that were previously conditioned in aerated river water for at least two weeks. Before the

start of the feeding experiment, organisms were starved for 48 h to ensure standardised hunger state.

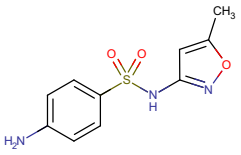
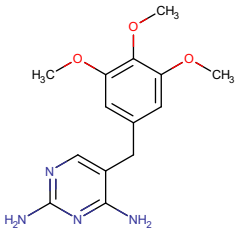
Table 4.1. River water parameters.

pH	T (˚C)	Total Hardness (as $CaCO_3$) (mg/L)	Nitrate (mg/L)		Total Alkalinity (mg/L)	Conductivity (uS)	Phosphate (mg/L)	Dissolved O_2 (mg/L)
			30 sec	60 sec				
8.8	10.9	425	0	2<x<5	80	500	<1	~ 8.7

4.3.2 Chemicals

SMX (CAS Number: 723-46-6) and TMP (CAS Number: 738-70-5) were purchased from Sigma-Aldrich (United Kingdom), (Table 4.2).

Table 4.2. Physio-chemical properties of the studied antibiotics. Source (www.drugbank.ca).

	Molecular structure	Molecular formula	Molecular Weight (MW)	Purity	CAS Number
Sulfamethoxazole (SMX)		$C_{10}H_{11}N_3O_3S$	253.28 g/mol	≥ 98%	723-46-6
Trimethoprim (TMP)		$C_{14}H_{18}N_4O_3$	290.32 g/mol	≥ 98%	738-70-5

4.3.3 Leaf preparation

Naturally abscised but undecomposed *Alnus glutinosa* leaves were handpicked in fall 2016 from a single tree in Sir Harold Hillier Gardens, Romsey, UK (51°00’47.3’’N, 1°27’53.8’’W). The leaves were taken back to a laboratory at

the University of Portsmouth, air dried and stored at 18°C until required. Dried leaves were soaked in charcoal filtered tap water for 2 h in order to soften them. From each leaf 1.3 cm Ø discs were cut with a plunger cutter, avoiding the main veins. Leaf discs were subsequently dried in a GenlabPrime oven (Genlab Ltd, UK) at 60˚C for 24 h and weighed to the nearest of 0.1 mg. Two weeks prior to the start of the experiment, stream water was collected from the River Ems, Westbourne, UK (50˚51'40.3''N; 0˚55'42.9''W). Stream water with a mixture of SMX and TMP was used to naturally conditioned the leaf discs for 7 days, and stream water controls were also established (n=15 per concentration). SMX and TMP were added in an equal concentration (1:1) to the mixture, each with a nominal concentration of either 2 µg/L, 20 µg/L and 200 µg/L. After the initial 7-day period, the stream water and antibiotic inoculum were renewed and left for a further 7 days. After the two-week conditioning process, samples of the conditioning water were frozen in liquid nitrogen and stored in a -80°C freezer for bacteria abundance analyses, whereas each leaf disc was thoroughly rinsed in bottled water (Evian®) to remove possible antibiotics residues, photographed with a camera (Leica MC120 HD) mounted on a stereo microscope (Leica S8APO) and provided to the organisms for the feeding experiment.

4.3.4 Feeding rate

An experiment was undertaken in order to investigate the possible impact of a SMZ and TMP mixture on the feeding activity of *G. pulex*. Each treatment included 15 replicates, consisting in a *G. pulex* specimen in a polypropylene pot filled with 100 mL of bottled water (Evian®). Each organism was provided with one leaf disc that was conditioned in a mixture of the two antibiotics (SMZ and TMP), each with a concentration of either 2 µg/L, 20 µg/L or 200 µg/L. The experiment lasted 24 h at 15°C with a 12:12 hours light:dark cycle. At the end of the 24 h, the *G. pulex* were sacrificed by freezing at -20°C and the leaf discs were photographed again. The *G. pulex* and the leaf discs were then transferred into an oven and dried for 24 h at 60°C. When dried, *G. pulex* specimens and the leaf discs were weighed to the nearest 0.1 mg. The leaf disc before and after photographs were subsequently analysed using ImageJ (https://imagej.net) to calculate the area consumed (Appendix C).

In order to quantify area and/ or mass loss during the conditioning process and the experiment itself, 15 control leaf discs were also prepared. These replicates underwent the same process as the other leaf discs used in the experiment, but they were not fed to the organisms.

4.3.5 Fungal biomass analyses

Fungal biomass on the leaf material from the decomposition experiment was estimated based on the mass of ergosterol, a component of eumycotic cell walls (Gessner, 2005). Briefly, using alkaline methanol, ergosterol was extracted from freeze-dried leaf material and subsequently purified by solid-phase extraction (Sep-Pak® Vac RC tC18 500 mg sorbent; Waters, Milford, USA). Ergosterol concentration was quantified by high-performance liquid chromatography (HPLC; 1200 Series, Agilent Technologies, Santa Clara, USA) at a wavelength of 282 nm. Ergosterol concentrations were converted to fungal biomass assuming an average mycelia concentration of 5.5 mg ergosterol.g^{-1} fungal dry weight (Gessner & Chauvet, 1993).

4.3.6 Bacterial abundance

Conditioning water samples (500 µL) were defrosted and stained with 50 µg/L of Sybr Green I DNA dye (diluted with Potassium citrate) for one hour in the dark. After this time, samples were further diluted (1:1) and cell count was undertaken with a flow cytometer (CyFlow® Cube 8, Sysmex Partec GmbH). Bacteria abundance was then calculated with the following equation:

$$Bacteria\ per\ mL = cell\ count * \frac{1000}{Flow\ rate * time\ (\mathrm{s})} * Dilution\ Factor$$

4.3.7 Data analyses

G. pulex Feeding Rate (FR) was quantified either as Leaf Area Consumed (see Equation 1) or as Consumed leaf mass (see Equation 2).

(1) $FR = \frac{A_i*(CF_A) - A_f}{w*t}$ (a) $CF_A = \frac{\left[\sum\left(\frac{A_{cf}}{A_{ci}}\right)\right]}{n}$

where A_i is the initial area of the leaf disc (mm^2), A_f is the final area of the leaf disc (mm^2), w is the animal dry weight (mg), t is the feeding time (days) and CF_A is the leaf change correction factor, where A_{ci} is the initial area of the control leaf discs (mm^2), A_{cf} is the final area of the control leaf discs (mm^2) and n is the number of replicates.

(2) $FR = \frac{L_i*(CF_1) - L_f}{w*t}$ (b) $CF_1 = \frac{\left[\sum\left(\frac{C_f}{C_i}\right)\right]}{n}$ (Maltby et al., 2002)

where L_i is the initial dry weight of the leaf disc (mg), L_f is the final dry weight of the leaf disc (mg), w is the animal dry weight (mg), t is the feeding time (days) and CF is the leaf change correction factor, where C_i is the initial dry weight of the control leaf discs (mg), C_f is the final dry weight of the control leaf discs (mg) and n is the number of replicates.

The data were analysed using IBM SPSS (version 24). Data relating to differences in the leaf area consumed and consumed leaf mass were found to be severely skewed, and thus were tested using non-parametric statistics, and the Kruskal-Wallis test was applied. The same analyses were also performed to verify differences in the fungal biomass and bacteria abundance.

4.4 Results

No statistical difference was measured in the consumed leaf mass between the different concentrations (Kruskal-Wallis test: H(3)=6.299 *p*=0.098) (Figure 4.1), whereas there was a significant difference in the leaf area consumed (Kruskal-Wallis test: H(3)=8.194, *p*=0.042) (Figure 4.2).

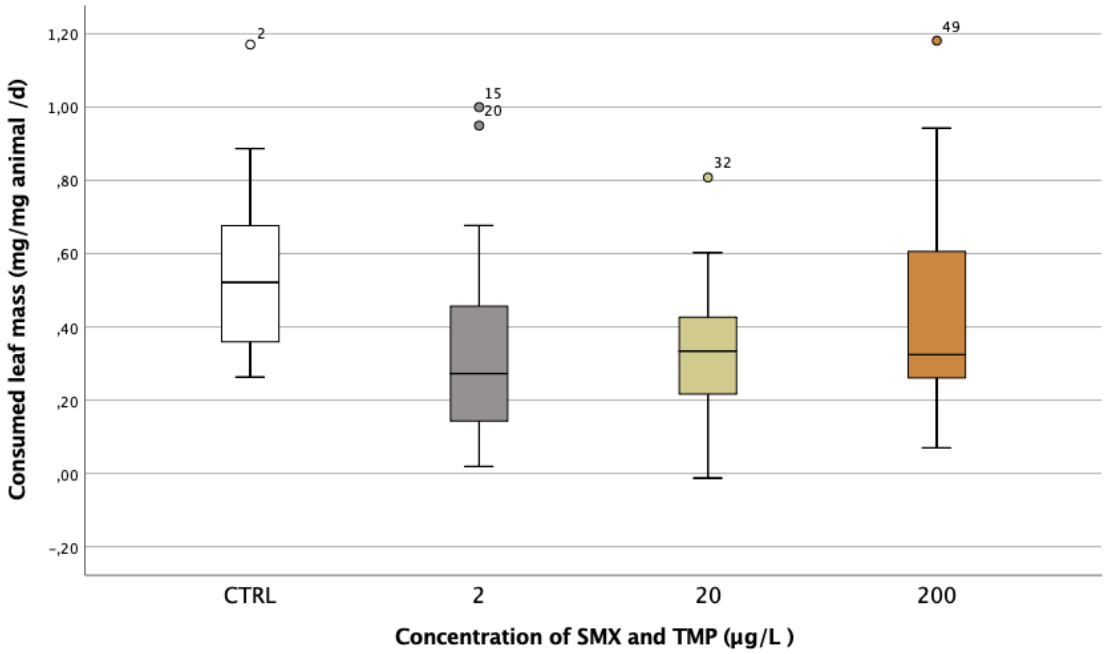


Figure 4.1. Consumed leaf mass by *G. pulex* when provided leaf discs that were conditioned in the presence of a mixture of the two antibiotics SMX and TMP. No significant difference was detected between the concentrations (Kruskal-Wallis test: H(3)=6.299, p=0.098). Lower and upper box boundaries represent 25th and 75th percentiles respectively. Line inside the box represents median. Lower and upper error lines represent minimum and maximum values respectively. Dots represent outliers. Outliers were included in the statistical analyses.

Pairwise comparisons showed a significant difference between the control and the 2 µg/L concentration (p=0.018) and between the control and 20 µg/L concentration (p=0.009), (Figure 4.2), indicating *G. pulex* specimens consumed a significant lower amount when provided with leaves that were conditioned in a mixture of 2 or 20 µg/L SMX and TMP compared to the control.

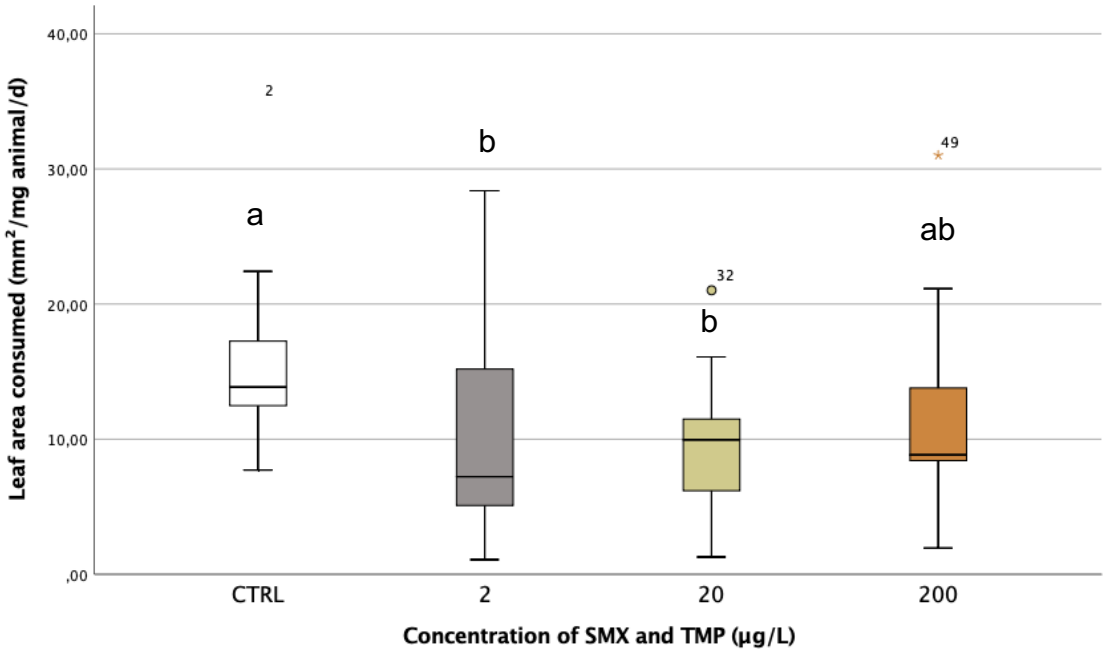


Figure 4.2. Leaf area consumed by *G. pulex* when provided leaf that were conditioned in the presence of a mixture of the two antibiotics SMX and TMP. There was an overall significant difference in leaf area consumption (Kruskal-Wallis test: H(3)=8.194, p=0.042). Pairwise comparisons detected a significant difference between the control and the 2 µg/L concentration (p=0.018) and between the control and 20 µg/L concentration (p=0.009). Lower and upper box boundaries represent 25th and 75th percentiles respectively. Line inside the box represents median. Lower and upper error lines represent minimum and maximum values respectively. Dots represent outliers and stars represent extreme outliers. Outliers and extreme outliers were included in the statistical analyses. Letters indicate significant differences between the different concentrations.

No significant difference was measured in the amount of fungal biomass associated with the control or any of the antibiotic conditioned leaves (Kruskal-Wallis test: H(3)=0.225, *p*=0.973) (Figure 4.3).

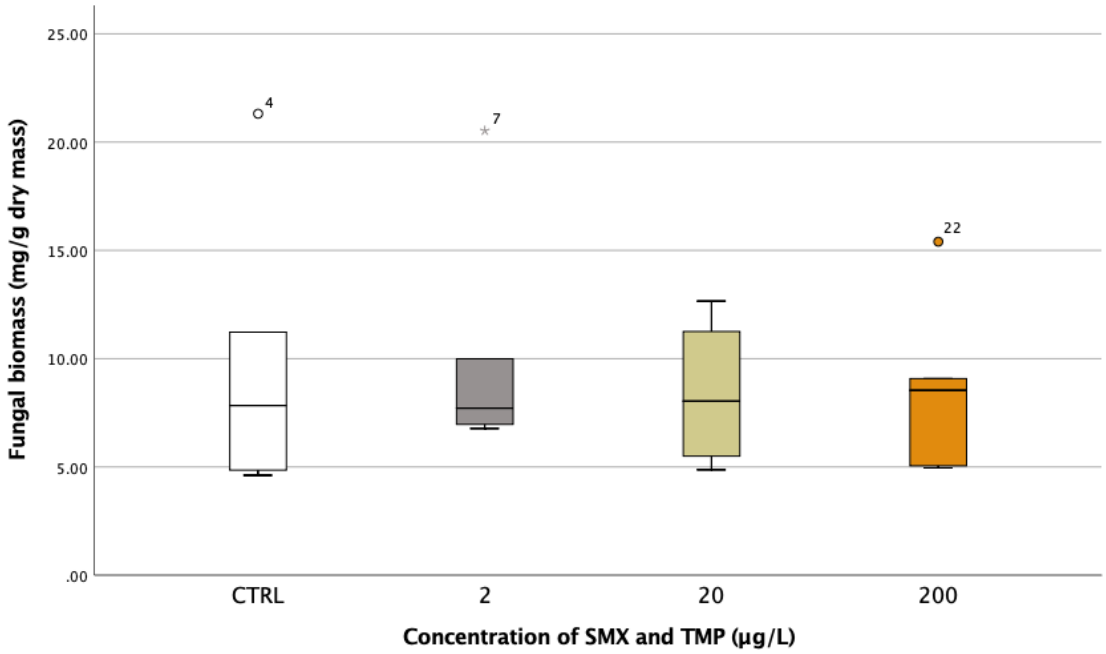


Figure 4.3. Fungal biomass associated with leaves conditioned in the absence (CTRL) and presence of a mixture of the two antibiotics SMX and TMP at different concentrations. No statistical difference was measured (Kruskal-Wallis test: H(3)=0.225, p=0.973). Lower and upper box boundaries represent 25th and 75th percentiles respectively. Line inside the box represents median. Lower and upper error lines represent minimum and maximum values respectively. Dots represent outliers and stars represent extreme outliers. Outliers and extreme outliers were included in the statistical analyses.

On the other hand, a significant difference was measured in the bacterial abundance in the conditioning water between the different concentrations (Kruskal-Wallis test: H(3)=19.244, p<0.001). Mean bacterial abundance in the water was found to be ~52% lower in the 20 µg/L (p=0.031) and ~58% lower in the 200 µg/L concentration (p=0.001) compared to the control (Figure 4.4), whereas no difference was measured with the lowest concentration (2 µg/L), (p=0.666).

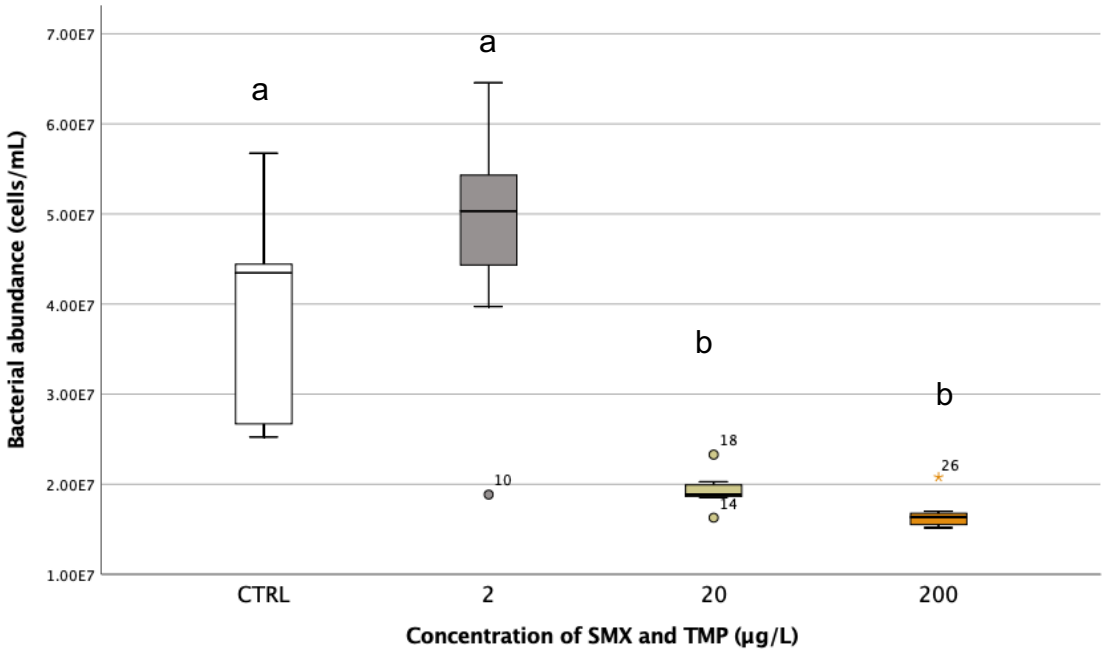


Figure 4.4. Bacterial abundance in the water used to condition the leaf discs. There was an overall significant difference in bacterial abundance (Kruskal-Wallis test: $H(3)=19.244$, $p<0.001$). Pairwise comparisons detected a significant difference between the control and the 20 µg/L concentration ($p=0.031$) and between the control and 200 µg/L concentration ($p=0.001$). Lower and upper box boundaries represent 25th and 75th percentiles respectively. Line inside the box represents median. Lower and upper error lines represent minimum and maximum values respectively. Dots represent outliers and stars represent extreme outliers. Outliers and extreme outliers were included in the statistical analyses. Letters indicate significant differences between the different concentrations.

4.5 Discussion

The authors initially hypothesised that the addition of antibiotics to the conditioning medium could interfere with the bacterial processes involved with leaf conditioning and the subsequent palatability of the leaf discs to the organisms. The presence of the SMX and TMP antibiotic mixture in the water was found to indirectly inhibit the *G. pulex* feeding rate, which showed a decreasing trend compared to the control, both as leaf area consumed and consumed leaf mass. However, the difference was found to be statistically significant at a concentration of 2 µg/L and 20 µg/L ,only when calculated as leaf area consumed.

In another study, Hahn & Schulz (2007) tested analogous concentrations of two separate bacteriostatic antibiotics, oxytetracycline (OCT) and sulfadiazine (SDZ), on the feeding rate of *G. pulex*. *G. pulex* feeding activity was measured as the leaf area consumed and was quantified as a food choice experiment. In that study, 13 specimens of *G. pulex* were simultaneously offered 5 leaf discs conditioned in either river water or river water spiked with OCT or SDZ. SDZ and SMX are part of the sulphonamide family and it was found that *G. pulex* ate a significantly less leaf material that was conditioned in the presence of either 20 µg/L OCT, 20 µg/L SDZ or 200 µg/L SDZ. Moreover, Hahn & Schulz (2007), in their study, found that a concentration of 2 µg/L OCT did not cause the gammarid feeding activity to deviate from the control.

Similar trends were identified in the feeding rate of *Gammarus fossarum* when exposed to several different fungicides (Zubrod et al., 2014). However, when the fungicides were included during the conditioning process no statistical difference was measured (Zubrod et al., 2015). Even though Gammarid feeding behaviour has been used for many years to investigate the potential impact of a variety of substances and to assess water quality, it can still be characterised by a pronounced intra variability, which could weaken the data and consequently the statistics (Consolandi et al., 2019).

SMX and TMP are characterised by a bacteriostatic mode of action (MOA), in other words these antibiotics will not ultimately kill the bacteria but only inhibit their growth by affecting the folate synthesis pathway. Consequently, one may speculate that the antibiotic mixtures used in this study might have interfered at an early stage, with the bacterial growth on the leaves and this could have possibly compromised the conditioning process and the palatability of the leaf discs. Bundschuh et al. (2009) obtained opposing results but as with Hahn & Schultz (2007), their experiment was set up as food choice trial. It was found that leaves conditioned in a complex mixture of five different antibiotics (erythromycin-H_2, roxithromycin, clarithromycin, trimethoprim and sulfamethoxazole) were more palatable, in particular those leaves that were conditioned with the highest concentration of 200 µg/L.

Bundschuh et al. (2009) also measured the fungal biomass as ergosterol content on the leaves surface. Contrary to our study, significant changes in the fungal

biomass were measured. It was suggested that the antibiotic mixture could have influenced the development of the bacterial communities on the surface of the leaves. Consequently, this could have allowed a shift in fungal abundance, making the treated leaves more palatable to the amphipods. The differences that this current study has found, in contrast to Bundschuh et al. (2009) could be explained by the different mixture adopted. Even though SMX and TMP were part of the early studies mixture, bactericidal antibiotics were also adopted (e.g. erythromycin-H_2, roxithromycin and clarithromycin). The presence of bactericidal agents might have allowed a higher fungi development on the leaf surface, which could have enhanced the palatability of leaf discs, by eliminating the bacteria. In the current study, the SMX and TMP mixture, especially at a concentration of 2 and 20 µg/L, might have had an effect on the leaf-associated bacterial communities that are involved in the conditioning process, which possibly led to less palatable leaves. Moreover, the quantification of bacteria abundance in the conditioning water showed that the median (20 µg/L) and highest (200 µg/L) concentration significantly reduced the total bacterial abundance. However, changes in abundance in leaf-associated bacterial communities were not quantified. Consequently, it is hard to confirm if the SMX and TMP mixture had the same effect on the bacterial communities in the water and on the leaf surface.

Even though Gammarids are biologically omnivorous organisms, they mainly feed on organic material, such as leaf litter and are characterised by a highly selective feeding behaviour. It has been demonstrated that they are able to choose between different leaf species (Bärlocher & Kendrick, 1973a; Friberg & Jaconsen, 1994) and also between unconditioned and conditioned leaves (Agatz & Brown, 2014; Graça et al., 1993a). Arsuffi & Suberkropp (1989) demonstrated how Gammarids are even capable of distinguishing between several fungi species. These selective behaviours appear to be based on physiological reasons. The organisms appear to select on what will promote higher growth and subsequent survival. Three different aspects have been suggested to influence Gammarids selectivity: (1) leaf toughness, (2) nutrient content and (3) plant secondary components (i.e. chemical defences) (Graça, 2001).

In the present study, only *Alnus glutinosa* leaves were used. However, the nutrient content depends not only on the leaf species, but also on the level of conditioning and on the fungi species. Leaf-surface communities of bacteria and

fungi produce different enzymes (e.g. pectinases, cellulases and xilanases), (Graça, 2001; Jenkins & Suberkropp, 1995) that facilitate the digestion of plant cell wall and consequently, freeing different compounds for the detritivores to assimilate. Moreover, organisms, such as aquatic hyphomycetes, are a source of polyunsaturated fatty acids (PUFA) for all those organisms that are incapable of synthesise them themselves. Invertebrates, and in particular crustaceans, cannot synthesise PUFA *ex novo* (Cornut et al., 2015; Saborowsky, 2015), but only convert a PUFA into another by elongation or desaturation. Therefore, Gammarids rely on their diet to assimilate PUFA. However, different fungi species are characterised by different PUFA concentrations (Cargill et al., 1985) and different degradative capabilities (Suberkropp et al., 1983; Butler & Suberkropp, 1986). Hence, even though there was no difference found in the ergosterol content, it is possible that different fungi species colonised the leaves to a different extent, making the different replicates more or less palatable. In the current study, leaves were conditioned by using natural river water. Consequently, the leaf discs could have been colonised exclusively by the spores that were present in the water phase and not by those that were on the leaves in the streams.

Lastly, one might speculate that the antibiotic mixture could have absorbed on the leaves' surface and potentially leaked back into the solution during the feeding experiment. Consequently, the observed differences in *G. pulex* FR could have been a direct or indirect response to the presence of antibiotics during the feeding trial.

Amphipods have a higher assimilation efficiency when fed conditioned leaves and/ or fungi (Bärlocher & Kendrick, 1975b; Graça et al., 2001; Kostalos & Seymour, 1976). Consequently, when in the environment, antibiotics could possibly alter the conditioning process, leaving detritivores with unpalatable and semi-unconditioned organic matter. This could have compromising consequences on the energy budget, growth and reproduction of Gammarids. Therefore, jeopardising Gammarid survival and more widely, impact the introduction of CPOM into freshwater ecosystems and other levels of the food web. However, in the current study the concentrations that had an impact on either the feeding rate or the bacterial abundance were above the concentrations reported in the environment, suggesting that environmentally relevant

concentrations of SMX and TMP might have a modest impact on the amphipods' feeding activity.

4.6 Conclusions

The current study suggested the mixture of the two bacteriostatic antibiotics SMX and TMP might have an impact on the feeding rate of the freshwater amphipod *G. pulex* when added to the water during the conditioning process at 2 and 20 µg/L. Even though an overall inhibition of the feeding rate was measured, this was not significant at the highest concentration of 200 µg/L. Similarly, bacterial abundance in water was statistically reduced at 20 µg/L and 200 µg/L compared to the control. The results suggest that at environmentally realistic concentrations (e.g. 2 µg/L), SMX and TMP might have an impact on the bacterial abundance in water or on the feeding rate of *G. pulex*. This could compromise the organisms' fitness, by affecting their growth and reproduction. Natural populations might be reduced in size, which would affect all those organisms that rely on Gammarids as a natural food source (e.g. amphibians, birds and fish). Additionally, the re-introduction of CPOM into freshwater environments would be impaired if a smaller number of shredding detritivores were present in natural ecosystems.

Chapter 5: The use of different behavioural methodologies to understand the effects of the antidepressant venlafaxine on the freshwater amphipod *Gammarus pulex*

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Author contribution:

All the data presented in this chapter were collected, processed and interpreted by Consolandi, G. The Multispecies Freshwater Biomonitor was provided by Gerhardt, A.. Statistical analyses were carried out by Consolandi, G. with the help and supervision of Parker, M.O. Consolandi, G. drafted the manuscript. Consolandi, G., Parker, M.O. and Ford, A.T. contributed and revised the final manuscript.

5.1 Abstract

The antidepressant venlafaxine (VEN) has been reported in wastewater effluents and in freshwater environments in North America and Europe in the range of ng/L to low μg/L. VEN belongs to the class of serotonin and norepinephrine reuptake inhibitors (SNRIs) and along with other psychoactive drugs it has been found to affect different functions in non-target organism. This study aimed to understand the impact of VEN on several behavioural endpoints (movement, ventilation, swimming velocity and feeding rate) in the freshwater amphipod *Gammarus pulex* and to understand the comparability of different behavioural methodologies for assessing ecotoxicity. *G. pulex* movement and ventilation were quantified by using the Multispecies Freshwater Biomonitor (MFB) after 11 days exposure to three environmentally relevant concentrations (0.02 μg/L, 2 μg/L or 20 μg/L). No significant difference in ventilation was measured, whereas organisms exposed to 0.02 μg/L were found to be more active (p=0.015) compared to the control. Swimming velocity was quantified by using the DanioVision™ observation chamber and it was found that a concentration of 20 μg/L induced a significant increase in swimming velocity after

7 days. A VEN concentration of 20 μg/L was also observed to induce a significant increase in the feeding rate of *G. pulex* after 24 h and 2 days. Both the MFB and the DanioVision™ observation chamber detected an increase in locomotion activity which is in line with previous studies in the literature. However, the lowest observed concentrations (LOECs) measured in this study were different depending on the methodology, which could have been the result of several differences (e.g. different organisms, different population, different seasons) in the experimental set-ups. Therefore, the experiments would need to be repeated in order to understand the effective sublethal ecotoxicity of VEN.

Keywords: Antidepressant, Behaviour, Feeding rate, *Gammarus pulex*, MFB, Movement, Swimming velocity, Venlafaxine, Ventilation.

5.2 Introduction

Over recent decades, aquatic ecotoxicologists have adopted a variety of different behavioural endpoints to understand the possible effects of contaminants, such as pharmaceuticals on freshwater organisms (Kramer et al., 1989: Gerhardt et al., 1998; De Castro-Català et al., 2017). The study of animal behaviour has proved to serve as a bridge between *ex situ* and *in situ* experiments (Pyle & Ford, 2017), as it can be used as a tool to integrate changes in physiology and biochemical processes in response to variations in environmental conditions (Gerhardt, 2007).

Sublethal behavioural endpoints are considered to be extremely sensitive and effective, as they are between 10 and 100 times more sensitive than acute tests (Robinson, 2009), and they can reveal the early stages of stress and impact on the organisms (Morgana et al., 2016). Consequently, behaviour could play an important role in better understanding the effective impact of low dose concentrations in the natural environment (Hellou, 2011).

Behavioural analyses are also less invasive and are usually more economical as for example they often don't require the use of expensive reagents (Bae & Park, 2014; Parker, 2016). However, behavioural ecotoxicology is often criticised and

underestimated due to problems with experimental repeatability, lack of standardisation and therefore, making comparison between different types of assays difficult (Alonso & Camargo, 2012; Melvin et al., 2017a; Parker, 2016; Sumpter et al., 2014). The undeniable development and improvement of new technologies has enabled increasingly sensitive, accurate and specific analyses (Bae & Park, 2014), such as photo and cutting-edge video tracking systems, to be undertaken (De Lange et al., 2006; Kohler et al., 2018).

The release of pharmaceuticals into the aquatic environment and more specifically the freshwater environment has been a concern for the scientific community but also the general public for several decades (Taheran et al., 2018). One of those pharmaceutical classes is antidepressants and in the last decade, the prescribing of these has increased dramatically and for some, such as venlafaxine, citalopram and sertraline has more than doubled (NHS, 2017). Venlafaxine (VEN) is an antidepressant belonging to the class of Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs) and it is often prescribed to treat depression and anxiety. SNRIs block the re-uptake of both serotonin and norepinephrine by pre-synaptic neurons that leads to a higher concentration of neurotransmitters in the synaptic gap and a greater number of post-synaptic receptors binding to the neurotransmitters (Lambert & Bourin, 2002).

VEN is considered one of the most commonly detected antidepressants in European streams (Bidel et al., 2016) and it is usually found in the ng to μg/L concentration range. González-Alonso et al. (2010) collected samples of river water downstream of ten different sewage treatment plants in Spain and found concentrations of VEN as high as 387 ng/L and a median value of 57 ng/L. In the United Kingdom VEN was detected in influent, effluent and river water with maximum concentrations of 343.8 ng/L, 269.6 ng/L and 71.6 ng/L (Baker & Kasprzyk-Hordern, 2011a). Water from influent and effluents of seven different English waste water treatment plants (WWTPs) were collected and analysed over a period of 12 months. VEN was detected in every sample and the final median concentrations were 141.2 ng/L in influents and 94.9 ng/L in effluents (Baker & Kasprzyk-Hordern, 2013). VEN was not detected in Portuguese river water, but it was detected in both influents (15.4 ng/L) and effluents (170.9 ng/L), (Paíga et al., 2017).

Outside of the European Union, VEN is also widely used and in the USA, Schultz & Furlong (2008) reported concentrations as high as 2190 ng/L in effluent water and 1310 ng/L in stream water. In New York state, mean concentrations were measured in influent up to 415 ng/L and effluent up to 480 ng/L (Subedi & Kannan, 2015). In South Africa, average concentrations of 94.6 and 35.4 ng/L, were measured downstream and upstream of a WWTP, respectively (Archer et al., 2017). Therefore, it is not surprising that several studies have focused on the ecotoxicological impacts of VEN.

VEN has been used in both acute and chronic experiments with the aim of understanding its possible impact on different endpoints. Schultz et al. (2011) exposed male fathead minnows (*Pimephales promelas*) to VEN for 21 days and discovered that it reduced survival up to 60% at an environmentally relevant concentration of 305 ng/L. On the other hand, in another study, fathead minnows were exposed to 3 different concentrations of VEN (0.88 μg/L, 8.8 μg/L and 88 μg/L) for their entire life cycle (167-168 days) and no statistically significant changes in survival or growth were observed, or in any of the other investigated endpoints (Parrott & Metcalfe, 2017). However, organisms that were exposed to 88 μg/L of VEN produced 46% more eggs per female and their eggs were not affected. In contrast, VEN was found to reduce the number of *Daphnia* offspring but the F1 generation was found to be tolerant to the antidepressant (Minguez et al., 2015).

Painter et al. (2009) exposed post-hatch fathead minnows embryos and larvae to several antidepressants (fluoxetine, sertraline, venlafaxine and bupropion), singularly and in a mixture, at three different environmentally realistic concentrations. It was found that VEN induced a delay in the latency period of both embryos and larvae. In another study, male mosquitofish (*Gambusia holbrooki*) were exposed to either 1 μg/L, 10 μg/L or 100 μg/L concentrations of fluoxetine, sertraline or venlafaxine. In the single antidepressant exposures, it was found that only 100 μg/L VEN concentration had a significant effect on the circadian rhythm of the fish (Melvin, 2017). The camouflage ability of the common cuttlefish (*Sepia officinalis*) was also reduced after a 20 day exposure to 0.1 μg/L VEN (Bidel et al., 2016) and after a 4 hour exposure to 157 μg/L VEN, it induced

foot detachment in 90% of the examined marine snails (*Chlorostoma funebralis*), (Fong & Molnar, 2013).

This study aims to identify if VEN impacts the behavior of the keystone species *Gammarus pulex* (*G*. *pulex*), [Linneus] and this will be evaluated by different methodologies. *G. pulex* is a freshwater amphipod, commonly distributed in freshwater streams in Europe and the British Isles (Karaman & Pinkster, 1977a), and because of its broad distribution, *G. pulex* has often been used as a test organism in ecotoxicological studies (Kunz et al., 2010). Among freshwater species, Gammarids are characterised by a high sensitivity to both organic and inorganic compounds (Wogram & Liess, 2001), so they are considered an effective test organism and indicator species to assess water quality. Studying the effects of VEN on *G. pulex* is important, because not only is it a noteworthy prey for fish and birds (MacNeil et al., 2000), but it also plays a key role in the decomposition of organic matter, so along with other species of Gammarids (e.g. *Gammarus fossarum*) it links different levels of the aquatic food web (Kunz et al., 2010).

The blood glucose of crustaceans is strictly regulated by the crustacean hyperglycemic hormone (CHH), which is itself regulated by numerous neurohormones (Fingerman, 1997a,b), such as serotonin and dopamine. Serotonin is a highly conserved monoamine (Robert et al., 2016). It is known to act as a neurotransmitter in crustaceans and to be involved in the regulation of glucose levels in several different crustacean species (Lorenzon et al., 2005; Robert et al., 2016) as it modulates the release of CHH (Lee et al., 2001). Moreover, previous studies have linked serotonin levels to changes in crustacean behaviour (Fong & Ford, 2014). Consequently, the authors hypothesised that VEN, an antidepressant of the class of SNRIs, could potentially alter *G. pulex* behaviour by affecting serotonin levels. To investigate this, changes in ventilation, movement, swimming velocity and feeding rate (FR) were investigated using different behavioural methodologies.

Gammarids ventilatory activity can be used as a sublethal endpoint as it indicates the organism's ability to provide oxygen to the gills and it is strictly connected to oxygen consumption and osmoregulation (Sormon et al., 2010; Wijnhoven et al.,

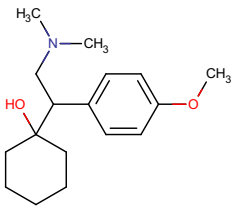
2003). Different stressors have been proven to affect ventilation in Gammarids such as heavy metals, temperature and acid stress (Felten et al., 2008a,b; Lawrence & Poulter, 1998; Sormon et al., 2010). Similarly, there are numerous studies reporting variations in locomotory activity after exposure to stress conditions and contaminants (Felten et al., 2008a,b; Funck et al., 2013; Lawrence & Poulter, 2001; Mehennaoui et al., 2016). Studying the possible effects on locomotion is important, because movement plays an ecologically relevant role in finding a mate, foraging and predator avoidance. FR is also considered an important sublethal endpoint as it is directly connected with on organism's survival, energy budget and fitness. Just as the ventilation and locomotory activity, the feeding rate of Gammarids has often been studied and quantified in response to stress conditions and exposure to pollutants (Blarer & Burkhardt-Holm, 2016; Consolandi et al., 2019; Ganser et al., 2019).

5.3 Material and methods

5.3.1 Chemicals

Venlafaxine hydrochloride (CAS number: 99300-78-4) was purchased from Sigma-Aldrich (United Kingdom), (Table 5.1).

Table 5.1. Physio-chemical properties of the studied antidepressant. Source (www.drugbank.ca).

	Molecular structure	**Molecular formula**	**Molecular weight (MW)**	**CAS number**
Venlafaxine hydrochloride		$C_{17}H_{27}NO_2 \cdot HCl$	277.4 g/mol	99300-78-4

5.3.2 Antidepressant exposure and Multispecies Freshwater Biomonitor (MFB)

The changes in movement and ventilation of *G. pulex* were investigated by using the Multispecies Freshwater Biomonitor (MFB), (LimCo International GmbH, Germany). The MFB is a device developed by Gerhardt et al. (1994) to track and record the multiple behavioural patterns of different aquatic organisms through a quadruple impedance conversion (QIC) technique. QIC is carried out as follows: each amphipod is placed in a plastic chamber (7.5 cm in length and 3.5 cm in diameter) that has two pairs of electrodes inside the wall: one pair produces a constant alternating current, whereas the second pair measures changes in the electrical field, which are the outcome of the organism's movements (Gerhardt et al., 1998). Changes in the electrical field are measured as changes in impedance of the system, which are then processed by a discrete Fast Fourier Transformation (Gerhardt et al., 2006) (Figure 5.1). Different types of behaviour and different organisms produce specific frequencies and the MFB measures the amount of time spent at each frequency (Gerhardt et al., 1994). Freshwater amphipods (e.g. *G. pulex*) produce low frequencies when swimming (from 0.5 to 2.0 Hz) and higher frequencies during ventilation (from 2.5 to 8.5 Hz), (Gerhardt et al., 2007). The MFB-data are expressed as cumulative percentage of time spent at a specific frequency.

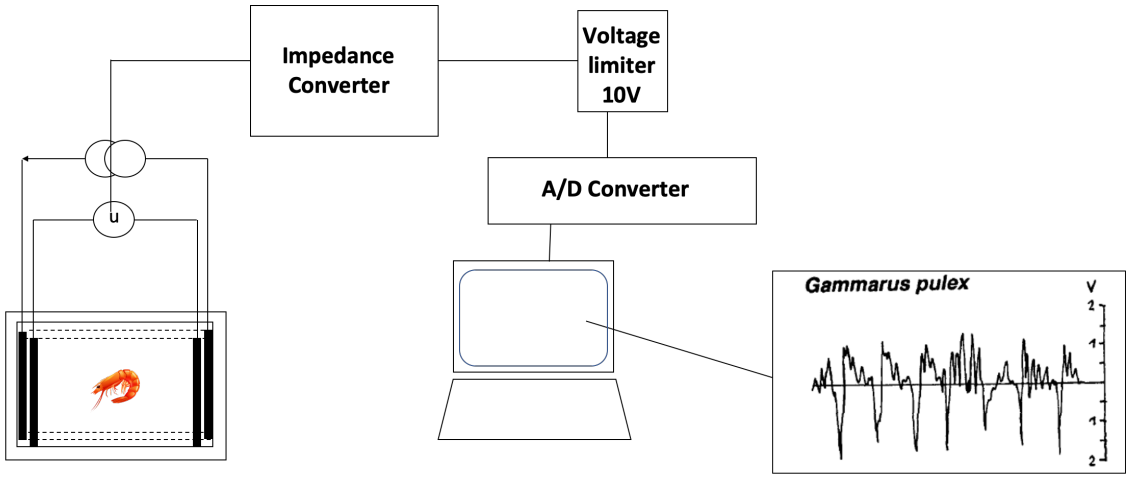


Figure 5.1. Components of the Multispecies Freshwater Biomonitor (MFB), (Adapted from Gerhardt et al., 1994).

5.3.2.1 Test organisms and acclimation

River water and specimens of *G. pulex* were collected from a non-polluted stream in Allensbach, Germany (47°42'27.6'' N, 9°06'26.5''E) in September 2017. The organisms were transported back to the LimCo International GmbH laboratory and acclimated for at least two weeks at 18°C in 20 L aerated aquarium filled with stream water and kept under dark conditions. The test animals were provided with alder leaves (*Alnus glutinosa)*, that were hand-picked in fall 2016 in the same location where the organisms were collected (Allensbach, Germany). The leaves were soaked for a week in filtered river water and air dried in the laboratory and stored until use.

5.3.2.2 Experimental set-up and the MFB

An 11-day serial exposure was set up to test the effects of three environmentally relevant concentrations of VEN (0.02 μg/L, 2 μg/L and 20 μg/L), starting from an initial stock of 20 mg/L. Each concentration included 6 replicates, consisting in 5 specimens of *G. pulex* in a glass beaker filled 100 ml of the filtered river water (for the experimental controls) or 100 mL of filtered river water with a nominal concentration of VEN of either 0.02 μg/L, 2 μg/L or 20 μg/L.

Into each beaker was placed with an *Alnus glutinosa* leaf disc (~ 3 cm ∅) and equipped with an aerating pump. Each beaker was placed into a controlled temperature chamber at 18°C in the dark, for the whole duration of the experiment. The control and test media were changed after 7 days.

G. pulex behaviour (movement and ventilation) was recorded five times over a period of 11 days by using the MFB. For each recording, 8 specimens were chosen from the 6 beaker replicates of each concentration. Each specimen was then placed alone in a chamber, which was situated in 4L aquarium filled with filtered stream water. The water in the aquarium was aerated before the start of the experiment, in order to prevent the aeration interfering with the electrical signal. Organisms were left 15 min to acclimate in the chamber, before their behaviour was recorded with the MFB for 2 hours. After the 2 h recording, each organism was placed back in its original beaker.

5.3.3 Feeding behaviour and the DanioVision™ experiments

Two separate experiments were set up to investigate the possible effects of the antidepressant VEN on *G. pulex.* The duration of the first experiment was 24 h and 7 days for the second experiment. For both experiments, changes in feeding rate and movement were investigated.

5.3.3.1 Leaf preparation

Freshly abscised but undecomposed black alder (*Alnus glutinosa*) leaves were handpicked in October 2016 from a single tree in Sir Harold Hillier Gardens, Romsey, UK (51˚00'47.3''N; 1˚27'53.8''W). The leaves were taken back to a laboratory at the University of Portsmouth, air dried and stored at room temperature in the dark until use. Dried leaves were soaked in charcoal filtered tap water for 2 h and subsequently 1.3 Ø cm discs were cut from each leaf with a plunger cutter, avoiding the main veins. Leaf discs were then dried in an oven at 60˚C for 24 h and weighed to the nearest 0.1 mg. Two weeks prior to the start of each experiment, individual leaf discs were conditioned in river water that was collected along with the organisms from the River Ems, Westbourne, UK (50˚51'40.3''N; 0˚55'42.9''W). After the two week conditioning process, the leaves were individually rinsed in bottled water (Evian®), photographed with a camera (Leica MC120 HD) mounted on a stereo microscope (Leica S8APO) and provided to the organisms for the feeding experiment (Appendix C).

For these experiments bottled water (Evian®) was used with the purpose of standardisation. Evian® is natural mineral water and it is mineral composition was in line (e.g. hard water) with the river water parameters from our designated collection site (Table 5.2 and 5.3).

Table 5.2. Evian® mineral composition in mg/L.

Mineral composition (mg/L)	
Sodium Na^+	6.5
Silicia SiO_2	15
Bicarbonates HCO_3^-	360
Sulphates SO_4^{2-}	14
Nitrates NO_3^-	10
Calcium Ca^{2+}	80
Magnesium Mg^{2+}	26
Potassium K^+	1
Dry residue 180°C	345 mg/L
pH	7.2

5.3.3.2 Test organisms

River water and *G. pulex* specimens (n=60) were collected two weeks' prior the start of each feeding experiment from the River Ems, Westbourne, UK (50˚51'40.3''N; 0˚55'42.9''W) using a hand-net. Parameters of the river water (total hardness, nitrate, total alkalinity and phosphate) were determined with colourimetric test kits (CHEMets® and HACH®) (Table 5.3). Conductivity, pH and oxygen saturation were also measured (Table 5.3). Organisms infected with the acanthocephalan parasite (*Pomphorhynchus laevis*) were discarded, since this parasite has been proven to affect the feeding activity of its host (Pascoe et al., 1995). *G. pulex* were taken to the University of Portsmouth to acclimate the organisms to laboratory conditions. Adult males (mean dry weight = 5.62 ± 1.45 mg (Experiment 1) and 6.32 ± 1.66 mg (Experiment 2) were isolated and kept at 15±0.1˚C under a 12:12 light:dark cycle for two weeks in a 3 L aquarium filled with bottled water (Evian®; pH=7.2) (Table 5.2) and fed *ad libitum* with *Alnus glutinosa* leaves that were previously naturally conditioned in aerated river water for at least two weeks. After the two-week acclimation phase, the organisms were

starved for 48 h before the start of each experiment, in order to ensure a standardised hunger state.

Table 5.3. River water parameters.

	pH	T (˚C)	Total Hardness (as $CaCO_3$) (mg/L)	Nitrate (mg/L)		Total Alkalinity (mg/L)	Conductivity (uS)	Phosphate (mg/L)	Dissolved O_2 (mg/L)
				30 sec	60 sec				
24 h experiment	7.4	-	425	0	5	240	580	<1	-
7 day experiment	7.5	13.9	425	0	2	240	594	<1	~ 8.5

5.3.3.3 DanioVision™

After 24 h (Experiment 1 and 2) and 7 days (Experiment 2), *G. pulex* swimming velocities were recorded using a 6-well plate (Kohler et al., 2018) in a Noldus DanioVision™ observation chamber connected to a Noldus EthoVision® XT 11.5 video tracking software (Tracksys, Nottingham, UK). Inside, the observation chamber was equipped with an infrared sensitive camera and a holder for a multiwell plate. Additionally, the holder can be backlit with a cold white light that can be programmed. After the 24 h (for Experiment 1 and 2), each organism was gently transferred with its medium from their experimental pot into one of the six wells of the 6-well plate. The 6-well plate was then placed in the Noldus DanioVision™ observation chamber, where organisms were left to acclimate to the new test conditions for one minute. The velocity (cm/s) of each specimen was recorded for 6 minutes, under a 3 minute dark: 3 minute light cycle with a 50% light intensity (2000 lx). A 3-minute dark: 3-minute light cycle was chosen in order to investigate the behavioural response of *G. pulex* to a sturbance (i.e. light) (Kohler et al., 2018). After this time, organisms were either sacrificed by freezing at -20°C (Experiment 1) or transferred back in their experimental pots for the rest of the experiment (Experiment 2). In the 7-day Experiment, each organism underwent the same process for a second time, before being sacrificed.

5.3.3.4 Feeding behaviour

The Experiment 1 investigated changes in the FR over 24 h, whereas Experiment 2 investigated changes in FR over a 7-day period by measuring changes in FR after 2 days, 5 days and 7 days exposure. In both experiments, three concentrations of VEN were tested. A stock solution with a nominal concentration of 20 mg/L of VEN was set up and then further diluted into the tested concentrations (0.02 μg/L, 2 μg/L or 20 μg/L). Each concentration included 15 replicates, consisting in one specimen of *G. pulex* in a polypropylene pot filled with 100 mL of bottled water (Evian®; pH=7.2), (for the experimental controls) or 100 mL of bottled water (Evian®; pH=7.2) with a nominal concentration of VEN, either 0.02 μg/L, 2 μg/L or 20 μg/L. For the 24 h experiment, each organism was provided with one leaf disc at 15°C in the dark. Following the 24 h feeding time and the behavioural analyses conducted with the Noldus DanioVision™ observation chamber, the *G. pulex* were sacrificed by freezing at -20°C and the leaf discs photographed (Appendix C). The *G. pulex* specimens and the leaf discs were then dried in a GenlabPrime oven (Genlab Ltd, UK) for 24 h at 60°C and subsequently weighed to the nearest 0.1 mg.

The 7-day experiment (Experiment 2) was also undertaken in the dark at 15°C. Each organism was provided with two leaf discs at the time that were replaced at day 2 and day 5, when water changes were due. Once removed from the polypropylene pot, each leaf disc was photographed again and dried at 60°C for 24 h and weighed to the nearest 0.1 mg. At the completion of the 7-day experiment (after FR and behavioural analyses), the *G. pulex* specimens were sacrificed by freezing at -20°C and dried in the oven for 24 h at 60°C, and subsequently weighed to the nearest 0.1 mg.

For each experiment, 15 control leaf discs were also established in order to calculate area and/ or mass loss during the conditioning process and the experiment itself. These control leaf discs underwent the same process as the other leaves used in the experiment, but they were not fed to the organisms.

5.3.4 Water analyses

5.3.4.1 MFB experiment

At the beginning of the antidepressant exposure, samples (100 mL) of each treatment dilution were collected, kept on ice and sent off the same day for analyses to DVGW-Technologiezentrum Wasser (Karlsruhe, Germany) in order to quantify VEN concentrations (Table 5.4) (Appendix D).

Table 5.4. Measured concentrations of VEN (μg/L).

Level of Instrumental detection	Nominal concentration	Actual concentration
0.010	0.02	2.5
0.02	2.0	1.5
2.0	20.0	16
2000	20000	16000

5.3.5 Data analyses

5.3.5.1 MFB experiment

The data obtained with the MFB were analysed using IBM SPSS version 24. A Univariate Analysis of Variance was applied for all the comparisons (type 1 error rate: α =0.05) and a Bonferroni correction was used for all post-hoc or pairwise comparisons and interactions.

5.3.5.2 Feeding behaviour and DanioVision™ experiments

G. pulex Feeding Rate (FR) was quantified either as Leaf Area Consumed (Equation 1) or as Consumed Leaf Mass (Equation 2).

(1) $FR = \frac{A_i * (CF_A) - A_f}{w * t}$ (Adapted from Hahn &Schulz, 2007),

(a) $CF_A = \frac{\left[\sum\left(\frac{A_{cf}}{A_{ci}}\right)\right]}{n}$,

where A_i is the initial area of the leaf disc (mm^2), A_f is the final area of the leaf disc (mm^2), w is the animal dry weight (mg), t is the feeding time (days) and CF_A

is the leaf change correction factor, where A_{ci} is the initial area of the control leaf discs (mm^2), A_{cf} is the final area of the control leaf discs (mm^2) and n is the number of replicates.

(2) $FR = \frac{L_i*(CF_1)- L_f}{w*t}$ (Maltby et al., 2002),

(b) $CF_1 = \frac{\left[\Sigma\left(\frac{C_f}{C_i}\right)\right]}{n}$

Where L_i is the initial dry weight of the leaf disc (mg), L_f is the final dry weight of the leaf disc (mg), w is the animal dry weight (mg), t is the feeding time (days). CF is the leaf change correction factor, where C_i is the initial dry weight of the control leaf discs (mg), C_f is the final dry weight of the control leaf discs (mg) and n is the number of replicates.

The data were analysed using IBM SPSS (version 24). Normality was first verified and then the significant area or mass loss was established either by Univariate Analysis of Variance (Experiment 1) or by a Linear Mixed Effects Model (Experiment 2), (Type 1 error rate: α =0.05) for all the different equations and comparisons, with exposure time (days) and concentration set as fixed factors and organisms' ID as random effect. A Linear Mixed Effects Model was also applied to estimate changes in the *G. pulex* swimming velocity (Experiment 1 and 2), with time (as seconds spent in the dark or in the light in DanioVision™ observation chamber), concentration and exposure (days; Experiment 2) set as fixed factors and organisms' ID as random effect. A Bonferroni correction was used for all comparisons and possible interactions (Experiment 2).

5.4 Results

5.4.1 MFB experiment

5.4.1.1 Movement

There was an overall significant difference in the organisms' movements between the treatments (Univariate Analysis of Variance: F(3,137)=4.363, *p*=0.006), (Figure 5.2A) (Table 5.5). There was also an overall significant effect of time (Univariate Analysis of Variance: F(4,137)=4.107; *p*=0.004) (Figure 5.2A)

(Table 5.5) meaning that over the 11 day period, the organisms were generally moving less. Pairwise comparisons showed a significant difference between day 4 and day 8 (Mean Difference (4>8): 352.896 ± 115.868, p=0.028) and between day 4 and day 11 (Mean Difference (4>11): 383.385 ± 115.868, p=0.012) (Table 5.6.).

Pairwise comparisons highlighted a significant difference between the control and the 0.02 μg/L concentration (Mean Difference (Control<0.02 μg/L): 319.217 ± 103.453, p=0.015) and between concentrations 0.02 μg/L and 2 μg/L (Mean Difference (0.02 μg/L >2 μg/L): 326.055 ± 103,453, p=0.012) (Table 5.7). However, no significant interaction was found between the different treatments and time (Univariate Analysis of Variance: F(12,137)=1.092, p=0.372) (Table 5.5) which means that during the experiment, the organisms generally moved less over time across the treatments.

Table 5.5. Univariate Analysis of Variance of *G. pulex* movement (n=120) after 11 days exposure to VEN.

Dependent variable: movement				
Source	**df**	**Mean square**	**F**	***p***
Concentration	3	921007.211	4.364	0.006
Time	4	866697.258	4.107	0.004
Concentration*Time	12	230357.521	1.092	0.372
Error	137			

Table 5.6. Pairwise comparisons for *G. pulex* movement at different exposure times (0 days, 4 days, 6 days, 8 days and 11 days).

Dependent variable: movement				
Exposure (I)	**Exposure (J)**	**Mean difference (I-J)**	**Std. Error**	***p***
0 days	4 days	-138.161	115.868	1.000
	6 days	-37.371	115.868	1.000
	8 days	214.735	116.880	0.683
	11 days	245.224	116.880	0.377
4 days	0 days	138.161	115.868	1.000
	6 days	100.790	114.847	1.000
	8 days	352.896	115.868	0.028
	11 days	383.385	115.868	0.012
6 days	0 days	37.341	115.868	1.000
	4 days	-100.790	114.847	1.000
	8 days	252.107	115.868	0.313
	11 days	282.595	115.868	0.160
8 days	0 days	-214.734	116.880	0.683
	4 days	-352.896	115.868	0.028
	6 days	-252.107	115.868	0.313
	11 days	30.489	116.880	1.000
11 days	0 days	-245.224	116.880	0.377
	4 days	-383.385	115.868	0.012
	6 days	-282.595	115.868	0.160
	8 days	-30.489	116.880	1.000

Table 5.7. Pairwise comparisons for *G. pulex* movement after exposure to different concentrations (CTRL, 0.02μg/L, 2 μg/L and 20 μg/L) of VEN over a period of 11 days.

Dependent variable: movement				
Concentration (I)	**Concentration (J)**	**Mean difference (I-J)**	**Std. Error**	***p***
CTRL	0.02	-319.217	103.453	0.015
	2	6.838	104.179	1.000
	20	-85.506	104.179	1.000
0.02	CTRL	319.217	103.453	0.015
	2	326.055	103.453	0.012
	20	233.710	103.453	0.153
2	CTRL	-6.838	104.179	1.000
	0.02	-326.055	103.453	0.012
	20	-92.344	104.179	1.000
20	CTRL	85.506	104.179	1.000
	0.02	-233.710	103.453	0.153
	2	92.344	104.179	1.000

5.4.1.2 Ventilation

G. pulex ventilation did not show any significant changes between the treatments (Univariate Analysis of Variance: F(3,136)=1.133, *p*=0.338) (Table 5.8). There was no significant difference in *G. pulex* ventilation over time (Univariate Analysis of Variance: F(4,136)=1.797, *p*=0.133) (Figure 5.2B). Also, no significant interaction between treatment and time was observed (Univariate Analysis of Variance: F(12,136)=1.713, *p*=0.070), which suggests that VEN did not affect the ventilation of *G. pulex* during the experiment.

Table 5.8. Univariate Analysis of Variance of *G. pulex* ventilation (n=120) after 11 days exposure to VEN.

Dependent variable: ventilation				
Source	**df**	**Mean square**	**F**	***p***
Concentration	3	1.544	1.039	0.377
Time	4	2.767	1.862	0.121
Concentration*Time	12	1.824	1.228	0.270
Error	136			

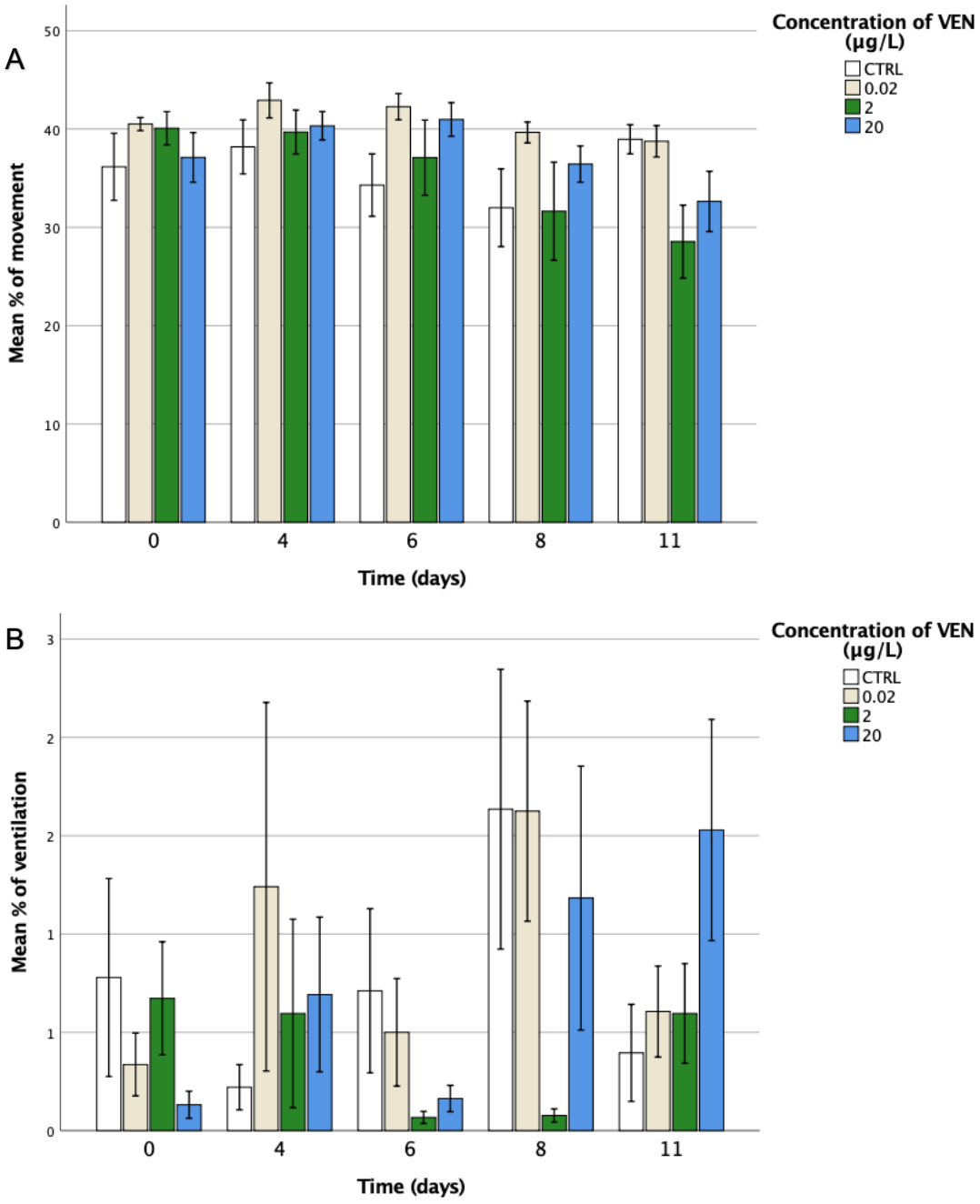


Figure 5.2. (A) Mean percentage of movement of *G. pulex* for each recording over a period of 11 days. (B) Mean percentage of ventilation of *G. pulex* for each recording over a period of exposure to VEN of 11 days. Data are expressed as mean ± standard error.

5.4.2 Feeding behaviour and DanioVision™ experiments

5.4.2.1 Experiment 1: 24 h

5.4.2.1.1 Feeding behaviour

After 24 h exposure to VEN, *G. pulex* feeding rate showed a linear dose effect trend, meaning that FR increased with higher concentrations (Figure 5.3).

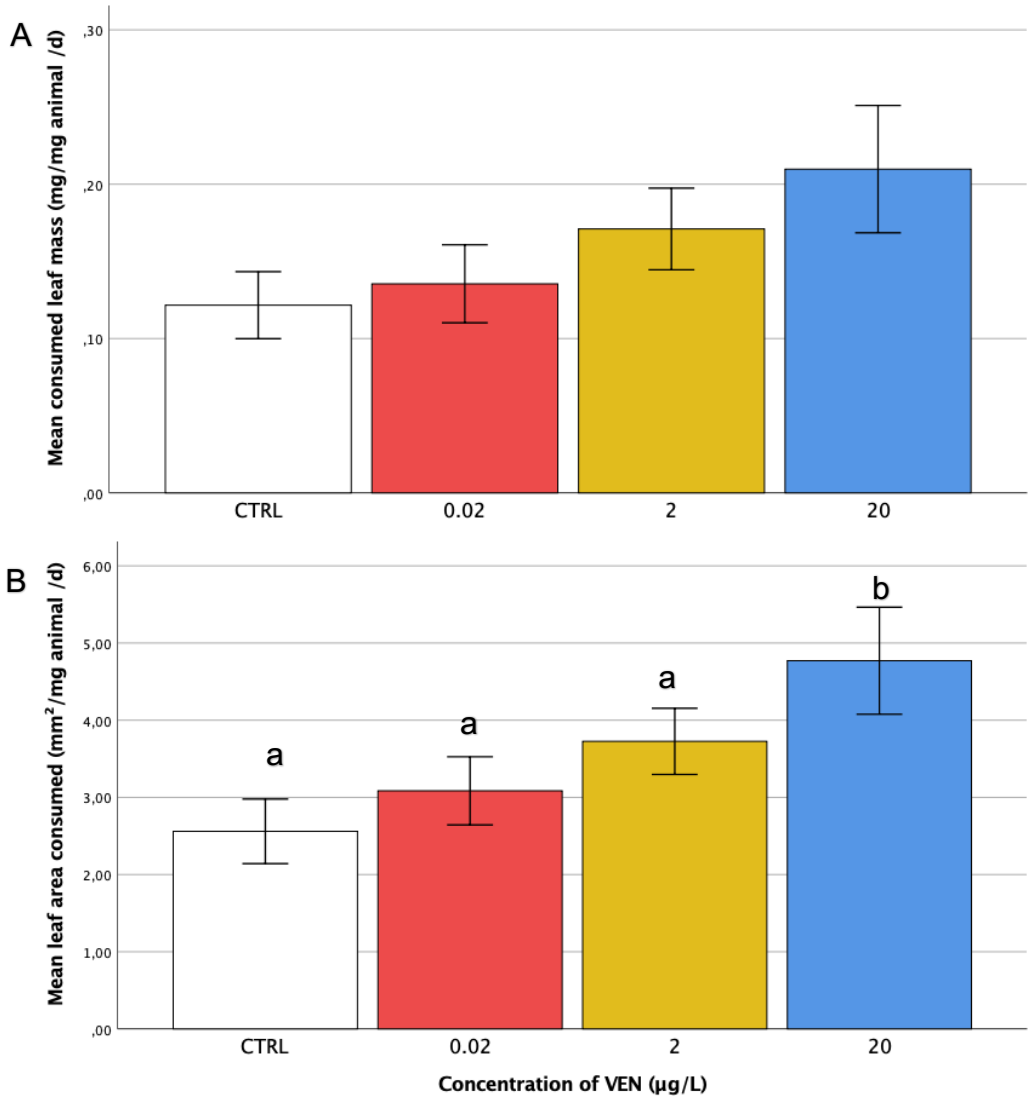


Figure 5.3. Mean feeding rate (± standard error) of *G. pulex* after 24h exposure to different concentrations of VEN. (A) Feeding rate expressed as mean consumed leaf mass. Differences in consumed leaf mass were not significant within the different concentrations (Univariate Analysis of Variance: F(3,51)=1.674, p=0.184). (B) Feeding rate expressed as mean leaf area consumed. A significant difference was measured within the different concentrations (Univariate Analysis of Variance: F(3,51)=3.322, p=0.027). Letters indicate significant differences between the different concentrations.

The amount of leaf mass consumed by *G. pulex* was not statistically different between the different treatments (Univariate Analysis of Variance: $F(3,51)=1.674$, $p=0.184$), (Table 5.9), (Figure 5.3A); however, when the FR was quantified as leaf area consumed a statistically significant difference was measured between the different concentrations ($F(3,51)=3.322$, $p=0.027$) (Table 5.9), (Figure 5.3B). In particular, multiple comparisons show (Table 5.10) that organisms exposed to the highest concentration of 20 μg/L consumed a significant larger amount of leaf area compared to the control (mean difference (Control<20 μg/L): 2.210 ± 0.75, $p=0.028$).

Table 5.9. Univariate Analysis of Variance of *G. pulex* feeding rate (n=55) after 24 h exposure to VEN.

	Source	**df**	**Mean square**	**F**	***p***
Dependent variable: leaf weight (mg/mg animal/d)	Concentration	3	0.021	1.674	0.184
	error	51			
Dependent variable: leaf area (mm²/mg animal/d)	Concentration	3	12.026	3.322	0.027
	error	51			

Table 5.10. Pairwise comparisons for *G. pulex* feeding rate after 24 h exposure to VEN.

Dependent variable: leaf area (mm²/mg animal/d)				
Concentration (I)	**Concentration (J)**	**Mean difference (I-J)**	**Std. Error**	***p***
CTRL	0.02	-0.545	0.74849	1.000
	2	-1.1654	0.73689	0.720
	20	-2.2105	0.74849	0.028
0.02	CTRL	0.5245	0.74849	1.000
	2	-0.6409	0.70704	1.000
	20	-1.6859	0.71913	0.138
2	CTRL	1.1654	0.73689	0.720
	0.02	0.6409	0.70704	1.000
	20	-1.0450	0.70704	0.873
20	CTRL	2.2105	0.74849	0.028
	0.02	1.6859	0.71913	0.138
	2	1.0450	0.70704	.873

5.4.2.1.2 DanioVision™

After 24 h exposure, no significant difference was found for the organisms' swimming velocities in any of the tested concentrations (Linear Mixed Effect Model: $F(3,56)=1.518$, $p=0.220$) (Figure 5.4) and similarly no significant interaction was detected between treatments and time (Linear Mixed Effect Model: $F(105,1960)=0.989$, $p=0.513$), meaning that regardless of treatment, the organisms reacted similarly to the light during the on and off phases. However, the organisms swam significantly faster when the light was turned on compared to when it was off (Linear Mixed Effect Model: $F(35,1960)=25, 473$, $p<0.001$), (Table 5.11).

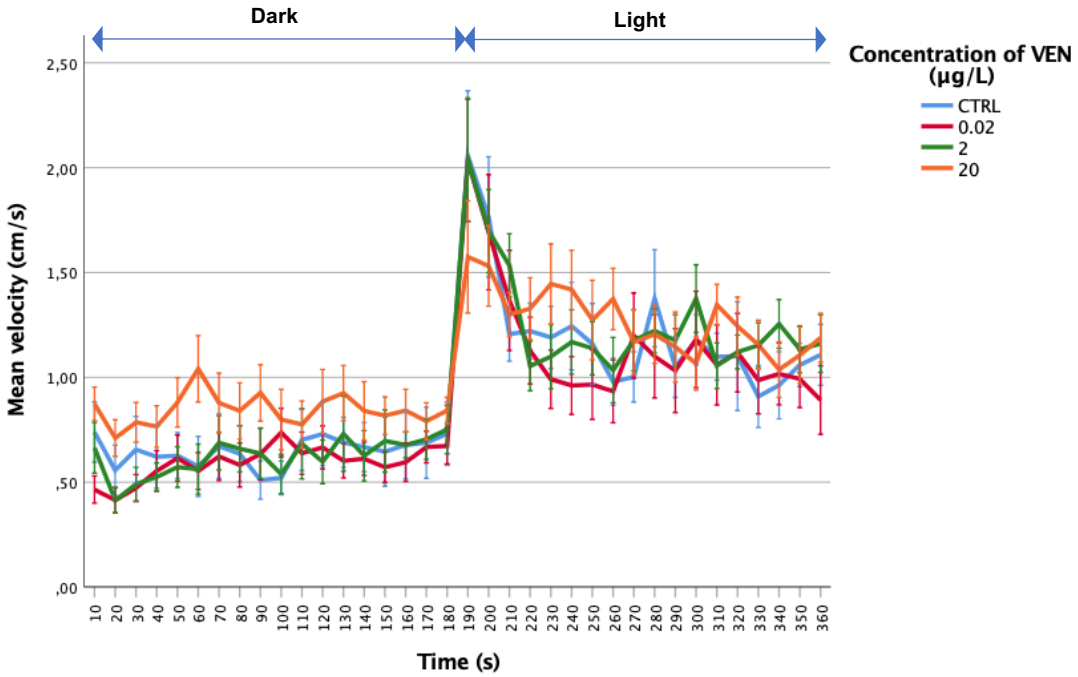


Figure 5.4. Mean velocity (± standard error) of *G. pulex* after 24h exposure to three different concentrations of VEN. No significant difference was measured between the different concentrations (Linear Mixed Effect Model: F(3,56)=1.518, p=0.220).

Table 5.11. Linear Mixed Effect Model of *G. pulex* velocity (n=60) after 24 h exposure to VEN. Concentration is to indicate the different concentration tested and time indicates the time spent inside the DanioVision™ chamber, namely 3 minutes dark: 3 minutes light photoperiod.

Dependent variable: swimming velocity (cm/s)				
Source	**Numerator df**	**Denominator df**	**F**	***p***
Concentration	3	56	1.518	0.220
Time	35	1960	25.473	<0.001
Concentration*time	105	1960	0.989	.513

5.4.2.2 Experiment 2: 7 days

5.4.2.2.1 Feeding behaviour

No statistical difference was observed in the consumed leaf mass between the different treatments (Linear Mixed Effect Model: F(3,38)=1.234, *p*=0.311) or the interaction between treatment and time (Linear Mixed Effect Model: F(6,76)=1.894, *p*=0.093) (Table 5.12) (Figure 5.5A). However, significant

differences were seen in the leaf area consumed between the different treatments (Linear Mixed Effect Model: F(3,38)=4.788, *p*=0.006) (Table 5.12), (Figure 5.5B). After 2 days, both the consumed leaf mass and the leaf area consumed show an increasing trend (Figure 5.5), which is similar to what was observed after 24 h (Figure 5.3). When *G. pulex* were exposed to a 20 μg/L concentration, they consumed a greater amount of leaf area compared to the control (Mean Difference (20 μg/L>Control): 0.472 ± 0.17, *p*=0.043) and the 0.02 μg/L concentration (mean difference (20 μg>0.02 μg/L)= 0.495 ± 0.16, *p*=0.019). There was also a significant interaction between treatment and time (Linear Mixed Effect Model: F(6,76)=3.252, *p*=0.007), which means the organisms exposed to 20 μg/L or 2 μg/L of VEN ate more compared to the other treatments over the duration of the experiment.

Table 5.12. Linear Mixed Effect Model of *G. pulex* feeding rate over an exposure period of days to VEN.

Dependent variable	Source	Numerator df	Denominator df	F	*p*
leaf weight (mg/mg animal/d)	Concentration	3	38	1.234	0.311
	Exposure	2	76	20.812	<0.001
	Concentration*Exposure	6	76	1.894	0.093
leaf area (mm²/mg animal/d)	Concentration	3	38	4.788	0.006
	Exposure	2	76	63.284	<0.001
	Concentration*Exposure	6	76	3.252	0.007

Pairwise comparisons revealed that after 2 days, organisms exposed to the 20 μg/L concentration of VEN ate significantly greater amounts of leaf area compared to the control (mean difference (20 μg/L>Control): 0.730 ± 0.23, *p*=0.018) and compared to the 0.02 μg/L concentration (Mean Difference (20 μg>0.02 μg/L)= 0.685 ± 0.22) (Table 5.13). After 5 days there was no statistically significant difference in the amount of leaf area consumed between any of the treatments ($p > 0.05$). However, after 7 days organisms exposed to a 2 μg/L concentration of VEN were found to have consumed significantly greater amount

of leaf area compared to the control (Median Difference (2 μg/L>Control): 0.868 ± 0.24, *p*=0.005) and compared to the 0.02 μg/L concentration (Median Difference (2 μg/L>0.02 μg/L): 0.831 ± 0.23, *p*=0.005) (Table 5.13). Overall

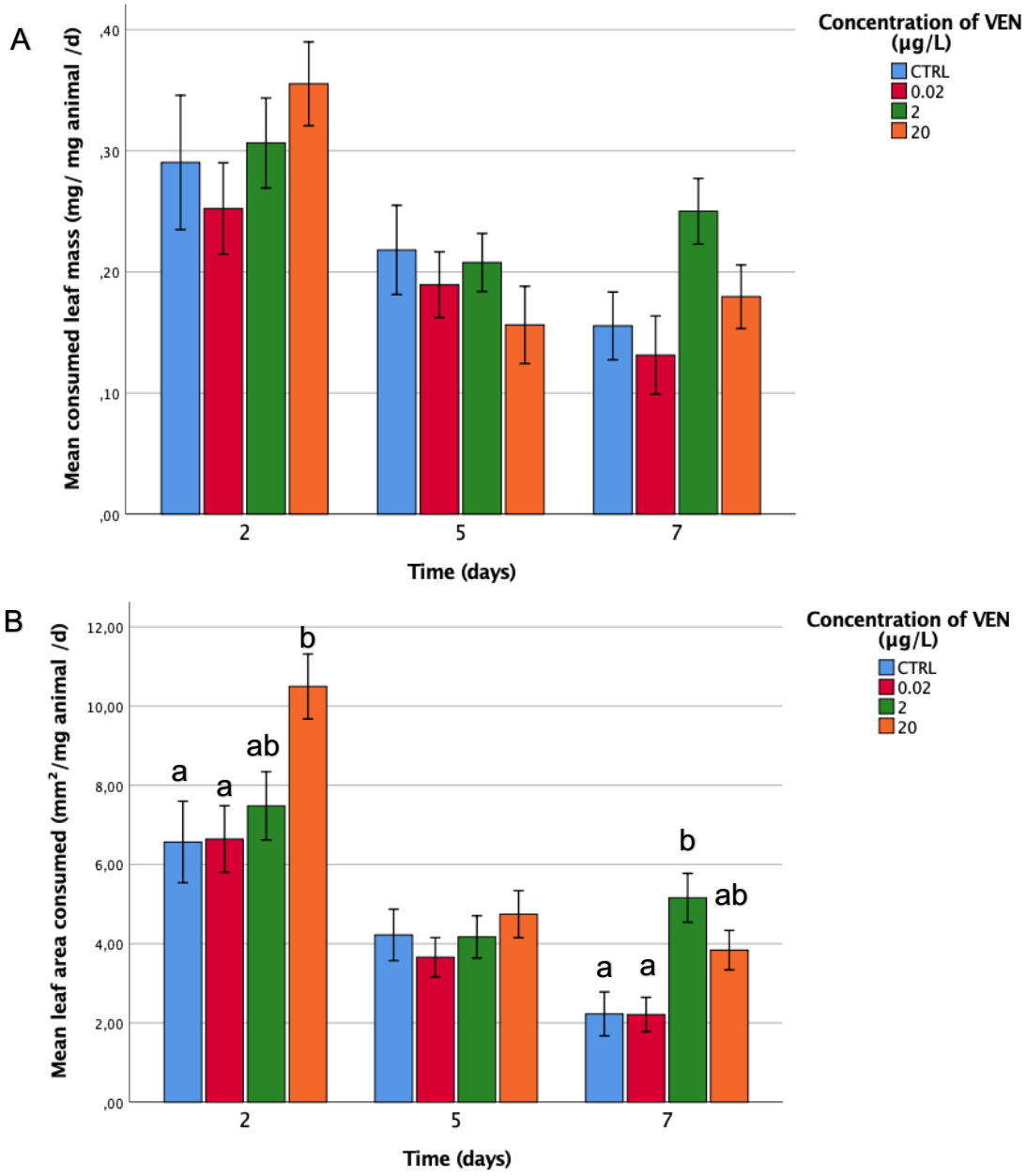


Figure 5.5. Mean feeding rate (± standard error) of *G. pulex* over a period of 7 days while being exposed to different concentrations of VEN. (A) Feeding rate expressed as mean consumed leaf mass. Differences in consumed leaf mass were not significant within the different concentrations over time (Linear Mixed Effect Model: F(6,76)=1.894, p=0.093). (B) Feeding rate expressed as mean leaf area consumed. Significant differences were measured within the different concentrations over time (Linear Mixed Effect Model: F(6,76)=3.252, p=0.007). Letters indicate significant difference between the different concentration within the same time of exposure.

statistically significant differences in the consumed leaf mass (Linear Mixed Effect Model: $F(2,76)=20.812$, $p<0.001$) and leaf area consumed (Linear Mixed Effect Model: $F(2,76)=63.284$, $p<0.001$) were measured over the length of 7 days exposure, which means that the *G. pulex* feeding rate generally decreased (Figure 5.5A & 5.5B) (Table 5.12).

Pairwise comparisons showed that the amount of leaf area eaten after 2 days was a significantly greater amount than after 5 days (Mean Difference (2>5): 0.737 ± 0.093, $p<0.001$) and 7 days (Mean Difference (2>7): 1.005 ± 0.093, $p<0.001$; Mean Difference (5>7): 0.268 ± 0.093, $p=0.015$) (Table 5.14). Similarly, it was found that the amount of leaf mass consumed by the *G. pulex* was significantly greater after 2 days compared to 5 days (Mean Difference (2>5): 0.108 ± 0.021, $p<0.001$) and 7 days (Mean Difference (2>7): 0.122 ± 0.021, $p<0.001$) (Table 5.14).

Table 5.13. Pairwise comparisons of *G. pulex* feeding rate after 2 days exposure and 7 days exposure to VEN. Concentrations are expressed in μg/L.

Exposure: 2 days					
Dependent variable: leaf area (mm²/mg animal/d)					
Concentration (I)	**Concentration (J)**	**Mean difference (I-J)**	**Std. Error**	**df**	***p***
CTRL	0.02	-0.045	0.235	38	1.000
	2	-0.209	0.240	38	1.000
	20	-0.730	0.230	38	0.018
0.02	CTRL	0.045	0.235	38	1.000
	2	-0.164	0.228	38	1.000
	20	-0.685	0.218	38	0.019
2	CTRL	0.209	0.240	38	1.000
	0.02	0.164	0.228	38	1.000
	20	-0.521	0.223	38	0.151
20	CTRL	0.730	0.230	38	0.018
	0.02	0.685	0.218	38	0.019
	2	0.521	0.223	38	0.151
Exposure: 7 days					
CTRL	0.02	-0.037	0.234	38	1.000
	2	-0.868	0.239	38	0.005
	20	-0.553	0.229	38	0.126
0.02	CTRL	0.037	0.234	38	1.000
	2	-0.831	0.227	38	0.005
	20	-0.516	0.217	38	0.136
2	CTRL	0.868	0.239	38	0.005
	0.02	0.831	0.227	38	0.005
	20	0.315	0.223	38	0.990
20	CTRL	0.553	0.229	38	0.126
	0.02	0.516	0.217	38	0.136
	2	-0.315	0.223	38	0.990

Table 5.14. Pairwise comparisons for *G. pulex* feeding rate measured at different exposure time (2 days, 5 days and 7 days).

Dependent variable	Exposure (I)	Exposure (J)	Mean difference (I-J)	Std. Error	df	*p*
leaf weight (mg/mg animal/d)	2 days	5 days	0.108	0.021	76	<0.001
		7 days	0.122	0.021	76	<0.001
	5 days	2 days	-0.108	0.021	76	<0.001
		7 days	0.014	0.021	76	1.000
	7 days	2 days	-0.122	0.021	76	<0.001
		5 days	-0.014	0.021	76	1.000
leaf area (mm^2/mg animal/d)	2 days	5 days	0.737	0.093	76	<0.001
		7 days	1.005	0.093	76	<0.001
	5 days	2 days	-0.737	0.093	76	<0.001
		7 days	0.268	.093	76	0.015
	7 days	2 days	1.005	0.093	76	<0.001
		5 days	-0.268	0.093	76	0.015

5.4.2.2.2 DanioVision™

Velocity was not significantly different between the treatments (Linear Mixed Effect Model: F(3,56)=0.934, p=0.430) and the organisms reacted similarly when the light was on and off across treatments (Linear Mixed Effect Model: F(105,3976)=1.209, p=0.074) (Table 5.15) (Figure 5.6A & 5.6B). However, animals swam significantly faster when the light was on compared to when it was off (Linear Mixed Effect Model: F(35,3976)=41.110, p<0.001), regardless of the concentration. A significant interaction was measured between treatment and exposure period (Linear Mixed Effect Model: F(3, 3976)=26.025, p<0.001), which means the organisms' swimming speed was different after 24 h and 7 days within the several treatments. Moreover, organisms swam significantly faster after 7 days compare to after 24 h (Linear Mixed Effect Model: F(1,3976)=292.623, p<0.001) (Figure 5.6A & 5.6B), and this was not related to the treatment. Pairwise comparisons showed that after 7 days exposure to VEN (Figure 5.6B) the

organisms exposed to the 20 μg/L concentration were significantly faster compared to the control (Mean Difference (20 μg/L >Control): 0.441 ± 0.15, p=0.021).There was also a significant interaction between time (lights on/off) and exposure (days) (Linear Mixed Effect Model: $F(35,3976)=8.935$, $p<0.001$), indicating that the organisms reacted differently when light came on after 24 h compared to 7 days (Figure 5.6A and 5.6B). Finally, a significant three-way interaction was also detected (time*treatment*exposure) (Linear Mixed Effect Model $F(105,3976)=1.250$, p=0.045), which indicates that the organisms reacted differently when the light came on, across the treatments and exposure periods (24 h and 7 days).

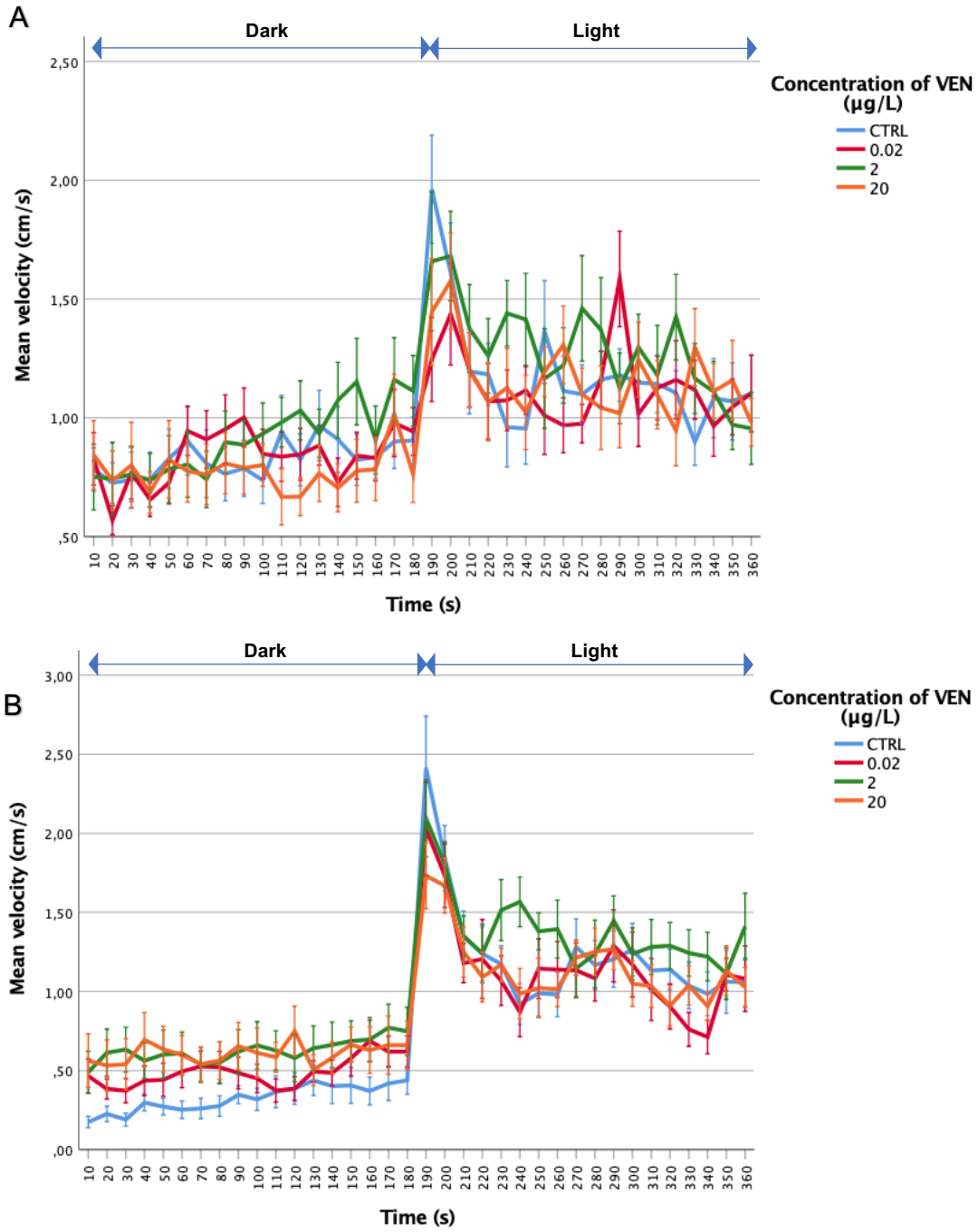


Figure 5.6. Mean swimming velocity (± standard error) of *G. pulex* after exposure to three different concentrations of VEN. (A) Mean velocity of *G. pulex* after 24h exposure. (B) Mean swimming velocity of *G. pulex* after 7 days exposure to the antidepressant VEN. Pairwise comparisons showed that after 7 days exposure organisms exposed to the 20 µg/L VEN swam significantly faster compare to the control.

Table 5.15. Linear Mixed Effect Model of *G. pulex* velocity (n=60) after exposure to VEN, measured after 24 h and 7 days. Concentration is to indicate the different concentration tested. Time indicates the time spent inside the DanioVision™ observation chamber, namely 3 minutes dark: 3 minutes light. Exposure indicates after how much time organisms' velocity was measured in the DanioVision™ chamber (i.e. 24 h and 7 days).

Dependent variable: swimming velocity (cm/s)				
Source	**Numerator df**	**Denominator df**	**F**	***p***
Concentration	3	56	0.934	0.430
Time	35	3976	41.110	<0.001
Exposure	1	3976	292.623	<0.001
Concentration*Time	105	3976	1.209	0.074
Concentration *Exposure	3	3976	26.025	<0.001
Time*Exposure	35	3976	8.935	<0.001
Concentration*Time*Exposure	105	3976	1.250	0.045

5.5 Discussion

In this study, amphipods were exposed to three different concentrations of the antidepressant VEN (0.02 µg/L, 2 µg/L, 20 µg/L). The 0.02 µg/L and 2 µg/L concentrations can be considered environmentally relevant as they fall within the range of concentrations that are currently measured in aquatic environments around the world (Baker & Kasprzyk-Hordern, 2011a; Archer et al., 2017).

In the first part of this study, *G. pulex* behaviour was studied and quantified by using the Multispecies Freshwater Biomonitor (MFB) to investigate changes in ventilation and movement. This experiment was carried out at LimCo International GmbH (Konstanz, Germany) in autumn 2017. The second part of the current study (feeding rate and swimming velocity) was instead undertaken at the University of Portsmouth (Portsmouth, UK) in spring and summer 2018. We were unable to bring the Multispecies Freshwater Biomonitor (MFB) in UK, consequently the second set of behavioural experiment was undertaken by using the DanioVision™ observation chamber.

In the second part of this study, design flaws were corrected: only one organisms was used for each replicate (e.g. Alonso et a., 2009; Bossus et a., 2014; Fent et al., 2006; Weber et al., 2018), only adult male organisms were selected (e.g. Guler & Ford, 2010), experiments were carried out in controlled bottled water instead of river water (Consolandi et al., 2019), river parameters were measured and experiment solution was renewed every 2-3 days instead of after 7 days.

*G. pulex v*entilation was not affected by exposure to VEN for 11 days. Although there was an overall decrease in movement behaviour in all treatments, a significant increase was observed in those organisms exposed to 0.02 µg/L compared to the control and 2 µg/L concentration. Samples of the aqueous antidepressant concentrations were analysed to quantify VEN concentration. The obtained values correspond to ~75% of the target nominal concentration and this is in line with previous studies using antidepressants (e.g. Minguez et al., 2015). However, the lowest nominal concentration of 0.02 µg/L was not confirmed and was instead reported to be 2.5 µg/L. Despite this, the movement behaviour of the organisms exposed to the supposed 0.02 µg/L concentration was noticeably different compared to the behaviour of those specimens that were exposed to 2 µg/L (actual concentration 1.5 µg/L). Moreover, each tested concentration was prepared through serial dilutions from an initial stock of 20mg/L. As all the other concentrations were confirmed by the water analyses, we believe it is plausible that an error took place during the quantification of VEN and that the data obtained at 0.02 µg/L are to be considered reliable.

Previous studies have already reported changes in locomotion activity in organisms exposed to antidepressants. De Lange et al. (2006) exposed specimens of *G. pulex* to the antidepressant fluoxetine at low concentrations (0.01 - 0.1 µg/L) using the MFB. De Lange and colleagues (2006) observed a significant decrease in activity, whilst organisms exposed to higher concentrations (1 µg/L - 1 mg/L) showed activity levels more similar to the controls. In a later investigation, *G. pulex* specimens swam significantly faster when exposed to fluoxetine concentration ranging from 1 µg/L to 1 mg/L (De Lange et al., 2009). Moreover, when two different species of marine snails were exposed to VEN, the results showed a significant increase in crawling speed to

reach the air-water interface, whereas the antidepressant fluoxetine tended to slow them down (Fong et al., 2015). However, this was only observed at concentrations that are at least 10 times higher than the highest concentration reported in the environment.

There are several studies in the literature where the antidepressant fluoxetine induces a significant increase in activity. Bossus et al. (2014) observed that the marine amphipod *Echinogammarus marinus* swam significantly faster compared to the controls after 1 day of exposure to 0.001 µg/L of fluoxetine and to 0.01 µg/L of sertraline, which, along with fluoxetine, is part of the selective serotonin re-uptake inhibitors (SSRIs). Moreover, *G. pulex* was exposed to 0.1 µg/L of fluoxetine and its swimming speed increased after a 14 day exposure (De Castro-Català et al., 2017). Mesquita et al. (2011) exposed specimens of the common shore crab *Carcinus maenas* to fluoxetine and they observed an increase in locomotion activity at concentration of 120 µg/L.

Interestingly, all these findings are consistent, not only with the MFB behavioural analyses, but also partially with the second part of the study, where the possible effects of VEN were assessed on the swimming velocity and the FR of *G. pulex* with two separate experiments (24 h and 7 days), and by using a different behavioural tracking device. VEN altered the *G. pulex* swimming velocity, in particular after 7 days of exposure. However, only organisms exposed to 20 µg/L VEN swam significantly faster compared to the controls. An increased activity rate could mean an increased predation threat and possibly a reduced level of fitness, due to a higher energy consumption required to sustain their increased activity (De Lange et al., 2006).

Glycogen is one of the most important metabolic macromolecules in Crustacea (Jimenez & Kinsey, 2015). It is synthesised from glucose that is the most abundant monosaccharide in the haemolymph and it is predominantly absorbed through the diet (Jimenez & Kinsey, 2015). Glycogen is broken down into glucose, which is then oxidised to produce ATP through glycolysis. During elevated exercise there is an initial release of the Crustacean Hyperglycaemic Hormone (CCH), followed by an increased concentration of glucose (Hoelters et al., 2016; Webster et al., 2012; Webster, 2015). Serotonin has also been

demonstrated to promote the release of CHH and consequently, induce hyperglycaemia in various Crustacea.

Specimens of the shrimp *Palaemon elegans* (Decapoda, caridae) injected with serotonin were found to have a significant higher concentration of CHH in their haemolymph, that was quickly followed by hyperglycaemia (Lorenzon et al., 2005). Similarly, injections of serotonin and/or fluoxetine induced significant hyperglycaemia in the crab *Chamsmagnathus granulata* and the crayfish *Orconectes limosus.* Moreover, both serotonin and fluoxetine induced a significant increase in the amount of circulating CHH in *O. limosus* (Santos et al., 2001). McPhee & Wilkens (1989) observed that serotonin injections induced alterations in the photonegative behaviour of the common shore crab *Carcinus maenas,* resulting in the organisms spending significant less time buried or hidden. In another study, specimens of *Carcinus maenas* were inject with either serotonin, fluoxetine or with a combination of both (Robert et al., 2016). It was observed that serotonin induced a quick increase in CHH and moulting inhibiting hormone (MIH) expression, whereas the response to fluoxetine was much slower. But both compounds induced also hyperglycaemia. Overall, these results seem to suggest that serotonin and antidepressants might be considered neuroregulators in crustaceans. Consequently, VEN could potentially induce an increase in the concentration of serotonin by inhibiting the serotonin transporters, and this could enhance the *G. pulex* activity rate. These two possible outcomes could together induce a boost in CHH release, which could be potentially be followed by hyperglycaemia.

Hyperglycaemia is the result of the simultaneous activation of phosphorylase and inhibition of glycogen synthase, leading to a depletion of glycogen and higher concentration of glucose (Santos & Keller, 1993; Sedlmeier, 1982). In order to sustain a higher activity rate (e.g. movements and/or swimming velocity), *G. pulex* will have to use glycogen and consequently glucose to power its movements. Consequently, a reduction in glucose levels could potentially lead to an increased feeding rate. In other words, the presence of antidepressants could lead to an increase in the activity of amphipods, that would result in lower levels of glycogen and consequently glucose.

In order to re-establish the glucose level in the haemolymph, Gammarids would need to increase their feeding rate. This hypothesis was confirmed in both experiments presented in this study (24 h and 7 days). The highest concentration of VEN (20 µg/L) was found to significantly increase the short-term (24 h and 2 day) *G. pulex* feeding rate. Whereas during a prolonged exposure (7 days), organisms were affected by the 2 µg/L concentration. In the long term, the 20 µg/L VEN concentration might have induced a more pronounced hyperglycaemia, and consequently could have inhibited the feeding activity.

One might speculate that this sequence could have detrimental repercussion at a population level, especially in those periods, when food sources are diminished, leading to higher competition and a higher risk of predation.

In this study, different behavioural methodologies have been used to investigate the possible impact that the antidepressant VEN might have on freshwater amphipods. However, the results obtained from the different experiments clearly highlight the uncertainty and high variability that are negatively associated with behavioural ecotoxicology. When the MFB was used, VEN was found to significantly enhance *G. pulex* movement behaviour at a 0.02 µg/L concentration, whereas by using the DanioVision™ observation chamber, VEN had a significant impact on swimming speed and feeding rate at a 20 µg/L concentration. These opposite outcomes might be the result of a combination of several factors, such as different endpoints, different acclimation and experimental temperatures, different countries and consequently different populations, different experimental set-up, different duration and different seasons. Therefore, it is difficult to make a comparison between the different methodologies and to draw a definite conclusion on the VEN concentration that might have negative effects on non-target organisms.

In the first part of the study, VEN appeared to induce a non-monotonic dose response, with a typical U-shape trend, in particular at day 6, day 8 and day 11. However, the control data showed a higher intra-variability and an unusual increasing movement trend at the end of the experiment (i.e. day 11) (Figure 5.2). Additionally, the same non-monotonic dose response was not observable in the second part of this study. Non-monotonic dose responses have recently been

associated with experiments using antidepressants at low concentrations (Bossus et al., 2014; Guler & Ford, 2010). However, these reports contradict other investigations where effects were measured after the exposure to high concentrations (Fong & Molnar, 2013; Fong et al., 2015). This tendency might have been a response to biological processes (e.g. moulting, age, sex) that this study was unable to estimate at the time due to limitations in the design. Consequently, the assumptions based on this data must be done cautiously, and in order to fully understand the potential effect of VEN, the experiments need to be developed and repeated.

In order to fully understand the ecotoxicological potential of VEN the present experiments would need to be carried out alongside biomarkers analyses. SSRIs and SNRIs have been demonstrated to affect the release of CHH and subsequent glucose levels in the haemolymph of several different crustaceans (Robert et al., 2016; Santos et al., 2001). Indeed, a hyperglycemia has been demonstrated to be a typical response of several crustaceans to environmental stressors (Lorenzon et al., 2005). The current study had originally included biomarkers analyses that aimed to quantify the total content of glucose in fully homogenised *G. pulex*. However, no data could be collected due to the limit of detection.

The lowest observed effect concentrations (LOEC) observed in this study were 0.02 µg/L during the MFB experiment and 20 µg/L for the feeding and swimming behaviour. Schultz & Furlong (2008) reported a VEN concentration in wastewater effluents in Minnesota of 2.19 µg/L and of 1.3 µg/L in a wastewater dominated stream, whereas VEN is usually detected in the ng/L range in modestly impacted riverine environments (Archer et al., 2017; Gracia-Lor et al., 2011). Thus, one of our LOECs falls into the range of environmentally relevant concentrations, whereas the second one is at least 10 times higher than the highest concentration measured in the environment. Nonetheless, these findings are important as they can be considered as an early warning, especially in those rivers that are deeply impacted and dominated by effluents discharge. VEN is nowadays one of the most prescribed antidepressants (Qu et al., 2018) and in the last decade the VEN prescriptions have more than doubled. Consequently, it can be speculated that VEN concentration in the environment could potentially increase even further and have a greater impact on non-target organisms. It has also been reported that

VEN can be bioaccumulated by different aquatic organisms (Arnnok et al., 2017; Bueno et al., 2014; Martínez-Morcillo et al., 2020; Qu et al., 2019).

Furthermore, additional investigation would need to be undertaken to focus on a range of different life stages. In the current study, adult organisms were used, but juvenile *G. pulex* are known to be more sensitive to pollutants. Consequently, repeating the investigation with juvenile *G. pulex* would help to better understand the possible impact that the antidepressant VEN could have on freshwater amphipods across their life cycle.

5.6 Conclusions

This study has provided evidence that the antidepressant VEN might have an impact on different behavioural endpoints in the freshwater amphipod *G. pulex*. A concentration as low as 0.02 μg/L was found to significantly increase *G. pulex* movement over a period of 11 days when quantified by using the MFB. Moreover, *G. pulex* FR was significantly affected by exposure to a VEN concentration of 20 μg/L. The same concentration induced also a significant increase in swimming velocity compared to the control. Impaired feeding rate, movement and /or swimming velocity might have broad ecological repercussion, by negativity affect fitness and population size, and consequently affect different level of food web.

Behavioural analyses have been proven over the years to be a useful tool to understand and quantify the sublethal potential of different contaminants. However, this study also highlighted how behavioural methodologies could lead to different conclusions, by over- or underestimating the toxicological potential of a specific pollutant. Therefore, future studies should try performing the same experiment multiple times and when possible, using different behavioural methodologies. It is recommended that behavioural studies include also specific biomarkers analyses, in order to better understand the real effective impact that a substance may have. Finally, different antidepressants have been shown to affect organisms in contrasting ways, consequently multiple antidepressants, belonging to either SNRIs or SSRIs, should be tested.

Chapter 6: General discussion and conclusions

6.1 Research summary and novel findings

The presence of pharmaceuticals in the aquatic environment is a problem that has increasingly held the attention of the scientific community and the general public over the last 40 years (Hignite & Azarnoff, 1977; Richardson & Bowron, 1985). The lack of legislation regarding the release and thresholds for environmental concentrations of these pollutants is likely to lead to further contamination of the associated freshwater ecosystems (Gogoi et al., 2018). This will also be exacerbated by an increase in population size and by the increasing number of more affordable and accessible medications (Comber et al., 2018).

Pharmaceuticals are active ingredients designed to perform a biological function at a low dose. With the continuous release of pharmaceuticals through WWTPs effluents, aquatic non-target organisms are constantly being exposed to sublethal concentrations (Santos et al., 2010). However, it is only recently that research has focused on the potential effects that environmental relevant concentrations may have on the aquatic fauna, emphasising the importance of adopting sub-lethal endpoints, instead of the more conservative acute tests (LC_{50} or LD_{50}).

The overarching aim of this PhD project was to try and understand if different classes of pharmaceuticals alter the behaviour of the freshwater amphipod *G. pulex*, with a specific focus on feeding behaviour, in order to evaluate its effectiveness and applicability as a sublethal endpoint in ecotoxicology. In addition, *G. pulex* swimming velocity and movement are also explored for use as potential sub-lethal endpoints, in the later chapters of the thesis (Chapters 3 and 5). The current piece of research provides evidence of unique novel findings that add to the present knowledge on feeding behaviour with *Gammarus* spp. but also on the prospective effects that certain pharmaceuticals may have on different behavioural parameters (e.g. movement, swimming velocity, feeding rate) at environmentally relevant concentrations.

The research was initially developed by critically reviewing and, for the first time, by systematically organising the published literature on freshwater *Gammarus* spp. feeding behaviour as a sublethal endpoint in ecotoxicology and identifying a number of variations within the published protocols (see Chapter 2; Consolandi et al 2019). The chapter concluded with several recommendations of how to strengthen and standardise feeding behavioural studies with Gammarids, so that research can be replicated and compared across the research community, but also be used to inform policy by regulators and other authorities. This paper was published in Reviews of Environmental Contamination and Toxicology (Chapter 2). Chapter 2 adds great value to the existing body of research on *Gammarus* spp. as species of the genus *Gammarus* are considered effective test organisms for ecotoxicological studies and their feeding activity has been shown to be representative of real environmental conditions (Maltby et al., 2002), making it a widely adopted sub-lethal endpoint to investigate the effect of different classes of pollutants. More interestingly, it was brought to attention how, at the moment, there are several different published equations in the literature that are used indiscriminately to calculate the feeding rate of *Gammarus* spp. (Agatz et al., 2014; Bundschuh et al., 2009; Coulaud et al., 2011; Geffard et al., 2010; Maltby et al., 2002).

Chapter 3 built on the research reviewed in Consolandi et al (2019) (Chapter 2) and focused on five different FR equations that have been applied to Gammarid feeding study data that is published in peer reviewed papers. The conclusion of Chapter 3 outlined that the different equations produced a different value for the FR when applied to the same data set and can therefore, lead to misleading results. Therefore, a compound's toxicity could be under- or overestimated. This can be considered an extremely useful discovery that could benefit feeding behavioural experiments by allowing more accurate inter-study comparisons. By interpreting the results obtained by the different equations, the ecotoxicity of the antidiabetic drug MET was also investigated, so the chapter also focused on the potential impact of that drug on FR and also the swimming velocity of the organisms. It was found that 0.1 μg/L, 1 μg/L or 10 μg/L concentrations did not have an impact on the swimming velocity of *G. pulex* during 24 h or 7-day studies, but it did have an impact on the FR of *G. pulex* after 2 days, but not after 24 h at

a concentration of 10 μg/L. This indicated that MET can have an impact in freshwater ecosystems, especially in those areas that are dominated by effluent discharge. Even though metformin has been on the market since the 1950's (Bailey, 1992), it has only been in the last few decades that researchers have been trying to understand if its extremely high environmental concentrations are a hazard for non-target organisms (Jacob et al., 2019; Lee et al., 2019; Niemuth & Klaper, 2018; Ussery et al., 2018). The ecotoxicological effects of MET have been studied on different aquatic animals and plants such as fish (Crago et al., 2016; Godoy et al., 2018, 2019; Jacob et al., 2018; Lee et al., 2019; MacLaren et al., 2018; Niemuth et al., 2015; Niemuth & Klaper, 2015, 2018; Ussery et al., 2018), amphibians (Melvin et al., 2017b), mussels (Koagouw & Ciocan, 2018), rotifers (García-García et al., 2017), aquatic plants (Godoy et al., 2018), algae (Cummings et al., 2018) and crustaceans (Markiewicz et al., 2017b). This is the first time that MET's toxicity has been evaluated on an amphipod and specifically on *G. pulex.* Studying the effects on *G. pulex* has an utmost ecological value, because of its key-role in the decomposition of organic matter in lotic environments and by being itself an important source of food for fish, birds and amphibians (Lebrun et al., 2019). The chapter also recommended the use specific FR equations for future studies as they produce the most accurate results and also relate to natural Gammarid feeding behaviour.

Chapter 4 moved the research forward on to look at pharmaceutical mixtures, with a particular focus on two antibiotics SMX and TMP (at 2 μg/L, 20 μg/L and 200 μg/L concentrations), in order to understand if these pharmaceuticals had an indirect impact on the *G. pulex* primary food source. *G. pulex* FR was significantly inhibited when specimens were provided with leaf discs that were conditioned in the presence of the 2 μg/L or 20 μg/L SMX and TMP mixture. The chapter concluded that the mixture might have had an impact on the *G. pulex* FR when added to the water during the conditioning. A concentration of 20 μg/L is although at least 10 times higher than the highest concentrations reported in the environment. A concentration of 2 μg/L can be considered more environmentally relevant, in particular in those water systems that dominated by wastewater discharge. Consequently, a 2 μg/L mixture of SMX and TMP may have an impact on the natural Gammarids populations.

The impact of the psychoactive pharmaceutical VEN was the focus of Chapter 5. This chapter examined the response of *G. pulex* to VEN and applied three different methods to examine the organisms response, in order to understand the comparability and sensitivity of several behavioural patterns: feeding behaviour, swimming velocity and movement. VEN induced a significant increase in movement at 0.02 µg/L, when measured with the Multispecies Freshwater Biomonitor (MFB). By using the DanioVision™ observation chamber, it was found that organisms exposed to 20 µg/L swam significantly faster after 7 days. Moreover VEN was also found to affect *G. pulex* FR after 24 h and 2 days at a concentration of 20 µg/L. Though there have been previous studies looking at the potential effects of antidepressants (e.g. fluoxetine) on Gammarids (e.g. De Castro-Català et al., 2017), this is the first time that the antidepressant VEN has been tested on *G. pulex* by comparing simultaneously different behavioural methodologies. The different methodologies (MFB and DanioVision™) were found to deliver different results regarding the effects on *G. pulex* locomotory behaviour. It was highlighted how the comparison is sometimes non-transferable and difficult, stressing the necessity to juxtapose behavioural endpoint with biomarkers (Coulaud et al., 2011). In this case especially, a full comparison between the methodologies adopted was not possible, due to essential differences between the experimental designs (see Chapter 5). The chapter concluded that the experiments would need to be re-run, in order to understand the effective concentration at which VEN may induce alteration in locomotion behaviour. Indeed, a concentration of 0.02 µg/L is environmentally realistic, whereas 20 µg/L is a least one order of magnitude higher than the maximum concentration reported in wastewater dominated streams. Nevertheless, both the MFB and the DanioVision™ identified an increase in *G. pulex* locomotion behaviour after exposure to VEN, which was associated with an increase FR.

Finally, this is the first piece of work adopting two different methodologies to estimate *G. pulex* feeding rate (one based on the weight of the leaves and the other one based on the differences in surface areas) and to draw attention to their comparability and interchangeability. This finding could potentially fill the current gap existing between feeding behavioural studies *in situ* and *ex situ* as the

methodology based on the changes in surface area is usually adopted for *in situ* investigations.

6.2 Overview of pharmaceuticals effects on *G. pulex*

G. pulex behavioural responses to the different tested pharmaceuticals are summarised in table 6.1. Each response was drug-specific, for example the antidiabetic MET induced a decrease in feeding rate whereas exposure to the antidepressant VEN stimulated *Gammarus* feeding activity. This different tendencies are not surprising, since each pharmaceutical used in this piece of work is characterised by a very different and specific mode of action. MET is involved in the regulation of blood glucose by increase the glucose uptake into cells and inhibiting hepatic gluconeogenesis and glycogenolysis. MET has also been proposed as a new medication to lose weight in non-diabetic obese people as it has an anorectic mode of action and it regulates appetite pathways (Seifarth et al., 2013). VEN inhibits the re-uptake of serotonin and norepinephrine in the synaptic gap and consequently a greater number of post-synaptic receptors will bind to the neurotransmitters (Lambert & Bourin, 2002). In crustacea, serotonin is involved in the regulation of glucose (Lee et al., 2001) and it has been proven to be connected with changes in behaviour (Fong & Ford, 2014).

Alteration in the feeding activity can have detrimental effects on the organism's fitness and consequent survival. Similarly, locomotion behaviour, if altered, may translate in damaging repercussions on the organism, and more widely on the entire population. In fact movement is essential for foraging, finding a mate and predator avoidance.

Table 6.1. Summary of the effects of the tested pharmaceuticals on the behaviour of *G. pulex*. ↑ and ↓ indicate a significant increase or decrease at the that concentration (µg/L), respectively. The symbol = indicates a response that was not significantly different from the control. N.A. stands for Not Applicable, as the endpoint was not tested.

Compound	**Class**	**Behavioural endpoints tested on *Gammarus pulex***				
		Feeding rate (based on the weight)	**Feeding rate (based on surface area)**	**Swimming velocity**	**Movement**	**Ventilation**
Metformin	Antidiabetic	↓ 10 (after 48h)	=	=	N.A.	N.A.
Sulfamethoxazole and Trimethoprim	Mixture of antibiotics	=	↓ 20 (after 24h) ↓ 2 (after 24h)	N.A.	N.A.	N.A.
Venlafaxine	Antidepressant	= = =	↑ 20 (after 24h) ↑ 20 (after 48h) ↑ 2 (after 7days)	= ↑ 20 (after 7days)	↑ 0.02	=

For each experiment, the possible relationships existing between the different endpoint tested have already been discussed in the corresponding chapter and compared with existing published literature. A direct relation between feeding rate and activity has already been reported in the literature (Felten et al., 2008a). In their study, Felten et al. (2008a) observed that a significant decrease in *G. pulex* feeding activity was associated with a reduction in the organisms' movement. This was not completely confirmed in our study. Increased feeding rate was seldom directly associated with an increase activity (e.g. swimming velocity) and this was observed not only with the antidiabetic MET, but also partially with the antidepressant VEN.

The antidiabetic MET did not affect either the feeding rate or swimming velocity of *G. pulex* in the short 24 h exposure, whereas it significantly inhibited the feeding rate after 48h without compromising the organisms' activities. On the other hand, the antidepressant VEN enhanced the feeding activity of *G. pulex* in

both exposures (i.e. 24 h and 7 days), but, similarly to the antidiabetic exposure, swimming activity was not directly related with changes in feeding rate.

By examining Table 6.1, it is self-evident how the way the feeding rate is calculated (one based on the weight of the leaves and the other one based on the differences in surface areas) may lead to different conclusions (Table 6.1). For each experiment in this PhD project, the feeding rate was quantified in two ways. Interestingly, the two methods (i.e. by using the weight or the surface area) adopted to calculate the feeding rate were found not to be completely interchangeable. This problematic has already been discussed in Chapter 3 in regards of the antidiabetic experiment. However, this is even more evident now by looking at the summarised results in Table 6.1. Indeed, even if in an opposite way, in the antidepressant trials the *G. pulex* feeding rate was impaired by exposure to VEN, however this was only statistically relevant as leaf area consumed, whilst the consumed leaf mass showed no differences compared to the control.

Calculating the feeding rate as leaf area consumed is most certainly a really accurate and precise method (Appendix C). However, this method is based on a single dimension and may not reflect accurately the loss in material. In fact, organisms might scrape the surface, but not enough for the image analyses to pick up the difference. Consequently, it would seem reasonable to discouraged the use of this method by opting for the more conservative and traditional method based on the difference in weight. However, this latter methodology was found to be characterised by an higher variability as a greater number of possible bias play a role in the data acquisition (e.g. scale precision, operator bias, level of dryness). Therefore, the decision to use both methodologies throughout the duration of this PhD project proved to be successful and enhanced the accuracy of our experiments and consequent data analysis and interpretation.

Each of the pharmaceutical tested in this project had, at some level, an impact on the feeding rate of *G. pulex*. However, not all of them pose the same risk and environmental hazard, simply because of the concentration they induced the effect at. The antibiotic mixture of SMX and TMP was found to indirectly alter *G. pulex* feeding behaviour at a nominal concentration of 2 µg/L and 20 µg/L, the

latter one, however, is at least 10 times higher than the majority of concentrations reported in the environment (see Table 1.1 in Chapter 1). On the other hand, the antidiabetic MET is usually measured in the aquatic ecosystems at really high concentrations (Table 1.1), consequently our results have a more prominent and crucial implication.

The antidepressant VEN induced effects at different concentrations, depending on the behavioural methodology adopted. When the Multispecies Freshwater Biomonitor (MFB) was used, *G. pulex* movement increased significantly in organisms exposed to 0.02 µg/L compare to the controls. A concentration of 0.02 µg/L is extremely relevant, however in another experiment, venlafaxine impaired the feeding rate at 20 µg/L in the short-term and at 2 µg/L after 7 days. Moreover, swimming velocity was altered exclusively at 20 µg/L after 7 days. One may speculate that the MFB is a more sensitive tool than the DanioVision™, however multiple variables may have played a role in the final outcome such as different acclimation temperature, different populations, different experimental set up, different seasons, moulting, age, sex. In fact, other studies have looked at the effects of the antidepressant fluoxetine by using either the MFB (De Lange et al., 2006) or the DanioVision™ (De Castro-Català et al., 2017). De Lange et al. (2006) concluded that a concentration range of 10-100 ng/L of fluoxetine significantly decreased *G. pulex* movement, whereas De Castro-Català (2017) observed an increase in velocity at 100 ng/L of fluoxetine.

VEN, contrary to other antidepressants (e.g. fluoxetine) has been reported in aquatic environments in concentrations up to 2 µg/L (Schultz & Furlong, 2008). Consequently, part of our results might have wider implications as very relevant and applicable to real environmental conditions, whilst other findings may be considered an early warning, in the unfortunate scenario of higher environmental concentrations.

6.3 Limitations of the current work

Behavioural endpoints have been often criticised in lacking repeatability (Melvin et al., 2017a) due to a high intra variability that, unfortunately, was also encountered during this PhD project. There are different factors that may have contributed in accentuating the aforementioned variability in each experiment. In

the following sections, problems that might have played a role in the outcome of our results will be critically analysed.

6.3.1 The organisms

Several characteristics of *G. pulex*, that are known to have an influence on variability (e.g. origin, age, sex), were taken into account. All the organisms used in this project, apart for the experiments conducted in Germany with the MFB, were collected from the same wild populations and even though wild organisms may be considered more or less sensitive to environmental stressors (Liber et al, 2007), they can provide a more realistic overview of the effects that certain contaminants may have on non-target organisms. However, their unknown life history may ultimately influenced their performance in behavioural analyses (Liber et al., 2007). Moreover, wild organisms may be pre-exposed to contaminants. Miller et al. (2019) investigated the presence of 107 different compounds (e.g. pesticides, pharmaceuticals and illicit drugs) in *G. pulex* specimens that were collected in 15 different sites in the UK. Out of the 107, 56 compounds were detected in the wild organisms.

G. pulex specimens were chosen of similar size (and consequently age) in order to limit the variability due to the use of different life stages. However, organism's exact size was measured (as dry weight) at the end of each experiment. In retrospect, as also suggested by Consolandi et al. (2019), (Chapter 2), organisms should have been precisely measured before the start of the experiment by measuring the dorsal length of their first thoracic segment (e.g. De Castro-Català et al., 2017). In fact, different life stages are characterised by contrasting sensitivities (Alonso et al., 2010; McCahon & Pascoe, 1998a) and it is possible that the variability could have been reduced if a narrower size range was chosen. However, this remains valid for adult organisms, as juveniles, even though are usually more sensitive to contaminants, have also been shown to be characterised by a higher inner variability in long-term exposures, making them more suitable for short testing (Agatz & Brown, 2014).

6.3.2 The conditioning process

Gammarus spp. are freshwater detritivores that usually feed on naturally conditioned organic material, especially leaf litter that reaches the aquatic environment from the surrounding riparian trees (MacNeil et al., 1997). The conditioning process refers to the colonization of organic material by bacteria and in particular by aquatic hyphomycetes (Bärlocher, 1985). In the published literature, there are three methodologies that are most commonly adopted: (1) conditioning the leaf material in small nets directly in the river stream (Danger et al., 2012); (2) conditioning the leaves in the laboratory by using river water and detritus that were previously collected (Hahn &Schultz, 2007); and (3) conditioning the leaf material in artificial river water inoculated with a specific fungi species (Bärlocher & Kendrick, 1973b).

In our case, in each experiment *G. pulex* specimens were fed alder leaves (*Alnus glutinosa*) that were conditioned in water that was previously collected from the same stream the organisms were sampled from. This method might be considered halfway between the others. The first method is logically the most environmentally realistic, however the leaf material may undergo contamination and be subjected to different weather conditions. On the other hand, the third method can be deemed to be the most standardised, but not reflecting the real environmental conditioning processes.

Aquatic hyphomycetes play a fundamental role in the decomposition and palatability of the leaf material. However, their colonization and propagation is dependent on the spores released in the water, the conidia (Bärlocher, 2009). In temperate streams, the number of conidia tends to sharply increase a few weeks after leaf fall in autumn, reaching concentrations up to 30000 conidia per litre, and markedly declines during winter and summer, when less than 10 conidia per litre may be present (Bärlocher, 2000, 2009). Consequently, conditioning leaf material in the laboratory with river water might not produce adequate and/or equally conditioned leaf material. Moreover, different species of fungi have been shown to be more or less palatable to *Gammarus* spp. (Arsuffi & Suberkropp, 1989). Therefore conditioning in the laboratory by using river water does not guarantee that the leaves will be colonised by the same species as they would if left *in situ*, and by the most palatable one.

6.3.3 Stress due to handling

Variability was encountered not only in feeding trials, but also in behavioural analyses carried out both with the DanioVision™ and the Multispecies Freshwater Biomonitor (MFB). The development of cutting-edge behavioural tracking devices has had a critical and noteworthy role in the implementation and improvement of behavioural ecotoxicology (Bae & Park, 2014), however organisms might still be subjected to handling stress.

The results obtained with the DanioVision™ equipment are an evident example of pronounced intra variability, especially in the first measurement after 24h (Chapter 3 and Chapter 5). It is possible that the organisms were still recovering from the handling stress of the day before, when the experiment was started, suggesting that there may have been the need for a longer period of recovery to the new experimental conditions.

The experiments conducted with the MFB were also characterised by noticeable variability, that, in this case, might have a been a direct response not only to a possible excessive handling, but also to underlying flaws in the experimental design. In fact, each replicate comprised 5 organisms in the same beaker and it cannot be excluded that it might have played a role in stressing the organisms. Moreover, because of the aforementioned set-up it was not possible to determine which organisms moulted and more importantly, males and females were used indiscriminately during the assay and even though gravid females were not selected, sex may have influenced their behaviour. Indeed, Peeters et al. (2009) studied the variation in locomotion behaviour in specimens of *G. pulex* by using the MFB and it was found that males were significantly more active than females amphipods.

Finally, the data recording with the MFB was conducted by placing the observation chambers containing the organisms in the same aquaria. Consequently, organisms from different replicates, opposite sex and possibly different treatments, were immersed simultaneously in the same media. This design led to the formation of pseudo-replications and probably enhanced the level of contamination and disturbance. Crustaceans can indeed communicate

with their conspecifics through the secretion of chemical substances (e.g. sex pheromones) (Subramonian, 2017; Thiel & Breithaupt, 2010).

6.3.4 Biomarkers analyses

Lastly, an important shortcoming of the current piece of research was the lack of biomarkers analyses. Behaviour is a response that interconnects changes in environmental conditions with possible undergoing physiological alterations (Pyle & Ford, 2017). Consequently, a multi-ecotoxicological approach might have helped in better explain and understand the repercussions that each tested pharmaceutical had on *G. pulex*, by highlighting the biochemical changes that are an indication of altered physiology.

6.4 Prospective work

The current piece of research reveals how each of the tested pharmaceutical compound can impair the feeding activity of *G. pulex*, validating the sensitivity of feeding behaviour as a sub-lethal endpoint. Nevertheless, a universal standardised methodology for feeding studies using *Gammarus* spp. still has to be reached and developed. To move forward, the impact that several parameters might have on feeding activity have to be investigated (e.g. temperature, sex, leaf species, acclimation duration, light:dark cycles) as they can have profound repercussions on the final results of an experiment and its consequent comparability with other studies (Consolandi et al., 2019). A first step in this direction has already been taken and it was proved that different feeding equations do not produce the same outcome (Chapter 3).

There are different prospective line of work that could originate from the novel findings in this PhD project. First of all, in order to have a deeper and clearer understanding of the hazard that the tested pharmaceuticals might pose to non-target organisms and in particular to *G. pulex*, the tested behavioural sub-lethal endpoints will have to be juxtaposed with biomarkers analyses that have to be chosen carefully, by taking into account the mode of action of each compound. Indeed, biomarkers analyses have been proven in the past to help elucidating

alterations in behaviour. For example, in their study, Gauthier et al. (2016) demonstrated how a mixture of copper and the polycyclic aromatic hydrocarbon phenanthrene can induce intense behavioural alterations (e.g. hyperstimulation causing uncoordinated movements) in the aquatic amphipod *Hyalella atzeca* by inhibiting the acetylcholinesterase activity (AChE).

The antidiabetic metformin appeared to decrease *G. pulex* feeding rate after 48h. In humans, metformin performs the action of reducing the production of glucose by inhibiting the hepatic gluconeogenesis and glycogenolysis (Viollet et al., 2012). Further work could potentially investigate the levels of glucose and glycogen in organisms that were previously exposed to the antidiabetic. Moreover, it has been suggested that metformin may act as endocrine disrupting compound (EDC) in fish (Niemuth & Klaper, 2018). Consequently, prospective work might also focus on investigating if the antidiabetic metformin might have similar effects on *G. pulex* , by looking a specific biomarkers such as the level of vitellogenin-like proteins, changes in the sex ratio or alteration in reproductive behaviour. Metformin is considered one of the top pharmaceuticals that is constantly discharged into the aquatic environment (Oosterhuis et al., 2013) and its consumption is expected to increase even further in the next decade (Armbruster et al., 2015). Therefore, future work might also investigate possible future scenarios with even higher concentrations.

The antidepressant venlafaxine appeared also to impair *G. pulex* feeding rate, consequently future investigations should include specific physiological analyses such as levels of the Crustacean Hyperglycaemic Hormone (CHH) and glucose. Indeed, over the years, antidepressants have been proven to act as neuroregulators in different crustacean species by inducing significant increases in the haemolymph levels of CHH and subsequent hyperglycemia (Santos et al., 2001).

Another line of work that could originate from this current piece of research is investigating the comparability of different behavioural methodologies.

Behavioural endpoints are sensitive, non-invasive and usually more affordable than other techniques (Hellou, 2011), however they are often criticised in lacking repeatability and in being characterised by high variability, making it hard to compare different methodologies looking at the same endpoint. Consequently, it would be extremely relevant for aquatic ecotoxicologists to be able to compare different studies, especially for *in situ* application. Moreover, the suggested approach could be applied on different life stages, as they are characterised by different sensitivities, which could translate in different behavioural responses.

6.5 Implications for the future

The development of a standardised feeding methodology and the improvement of the comparability between behavioural endpoints, could lead to a greater adoption of these methodologies in environmental risk assessments (ERAs) and in monitoring programs.

Indeed, since the Water Framework Directive (WFD) was issued on the 23rd of October 2000 (Council Directive 2000/60/EC), there have been significant achievements and improvements in policies regarding environmental quality status, highlighting once again the importance of safeguarding aquatic environments and their biodiversity (Brack et al., 2017). In this respect, the integration of behavioural assays along with biomarkers could translate in a more exhaustive method of detection. Since 2006, every medicinal product for human use has to be assessed for environmental risks by following the guidelines provided by European Medicines Agency (EMEA, 2006). The assessment is a two-phase procedure: in Phase I the predicted environmental concentration (PEC) for surface water is calculated. If the PEC value is equal or above 0.01 μg/L, then a Phase II is required and the substance properties (i.e. bioaccumulation, persistence and toxicity) are investigated. Phase II is characterised of two possible phases as well. In Phase II A, the predicted no-effect environmental concentrations (PNECs) are calculated based on standard toxicity test using fish, daphnia and algae. If the ratio between PEC and PNEC is above 1 than a Phase II B is carried out to further evaluate the substance toxicity in the environment (EMEA, 2006). The EMEA guidelines recommend the use of standard toxicity tests that follow the guidelines of the Organisation for Economic

Cooperation and Development (OECD Test Guidelines 201, 211 and 210). However, the OECD tests were initially developed to investigate the toxicity of industrial chemicals, whereas pharmaceuticals are specifically designed to perform a biological function and are characterised simultaneously by low toxicity (Ågerstrang et al., 2015). Therefore, OECD's toxicity tests may not be the most appropriate. Moreover, behavioural analyses (e.g. feeding activity, locomotion activity) are not part of standard ERAs, even though behavioural endpoints are considered ecologically relevant and they have been proved to be between 10 and 100 times more sensitive than traditional toxicity tests (Gerhardt, 2007). Additionally, behavioural tests are characteristically non-destructive and less-invasive than other methodologies and they usually more affordable, which would make them suitable and cost-effective as comprehensive monitoring-tools (Bae & Park, 2014). Because of all these reasons behavioural assays might be considered more "acceptable" by the public community, whilst being ecologically relevant (Robinson, 2009). The integration of behavioural endpoints in ERAs would allow a clearer and deeper understanding of the effective toxicity of certain contaminant, by further strengthening the results of standard tests. Feeding and locomotion, along with other behavioural patterns, can functions as early warnings as alterations could have profound repercussions on growth, survival and reproduction, and consequently on the entire population and ecosystem.

The integration of behavioural endpoints in ERAs would gain even more significance if conducted with other test species as different organisms are characterised by being more or less sensitive to different classes of contaminants (Wogram & Liess, 2001). *G. pulex* is a prominent example and it would fulfil the purpose as it is widely distributed in the Northern Hemisphere, it can be bred in laboratory conditions, it is sensitive to a wide range of contaminants and it plays a key role in freshwater ecosystems.

The scientific community is often attempting to provide valuable information about the toxicity of compounds to policy-makers and environmental agencies, in order to facilitate future action (Brack et al., 2017). However, there is still a great lack of knowledge about the possible effects that many contaminants, and in particular

pharmaceuticals, might have on aquatic ecosystems and non-target organisms, making it more difficult to put new legislations and restrictions in place.

A great challenge is the identification, and consequent assessment and management, of those pharmaceuticals that may pose the greater risk for the environment. A possible prediction method can be based on current and future consumption (Gómez-Canela et al., 2019). The antidiabetic MET is a prominent example of increased administration and use, that is not likely to decrease, especially in developed countries, where the rate of obese individuals affected by type II diabetes is constantly growing (Tiesler & Zwiener, 2018). Moreover, MET is a newly adopted dieting medication in non-diabetic obese people (Seifarth et al., 2013) and it has recently been proposed as a potential treatment for certain types of cancer (Daugan et al., 2016; Kasznicki et al., 2014). Therefore, metformin environmental concentration will rise even more. However, it is unlikely the prescription and use of metformin will be limited, as for many other essential pharmaceuticals.

Effluents are considered to be the major route of contamination for pharmaceuticals in the aquatic environment. Therefore, it is unsurprising that since the 1990s, the first point of action for pollution control has been focusing on finding means to reduce the entry of pharmaceuticals in the aquatic environment through treated sewage (e.g. the updating and modernization of WWTPs), (Daughton, 2014). However, this could be financially restrictive and it could take decades before completion. Moreover, the number of medications that are nowadays available on the market, falls into the range of thousands, making it even more difficult to conceive engineered technologies capable of treating and removing such a wide variety of different compounds (Jones et al., 2005).

There are numerous studies in the literature focusing on understanding which are the most effective treatment processes to remove pharmaceuticals residues from influent waters. However, it is clear that different processes may not be feasible for every compound or may lead to the formation of transformation product, which themselves can be toxic and biologically available (Kümmerer, 2019). Scheurer et al. (2012) investigated the fate of MET during wastewater treatment. It was found that flocculation and activated carbon filtration are ineffective to remove MET, whereas ozonation and chlorination partially remove MET, but also lead to

the formation of new transformation products (Armbruster et al., 2015). Riverbank filtration was found to effectively remove MET (Scheurer et al., 2012). Similarly, VEN is not completely removed by chemical and biological processes (Lajenusse et al., 2012) and that even after the application of ozonation, VEN is still present in the final effluent, along with its transformation products (Lajenusse et al., 2013; Zucker et al., 2018). Furthermore, the antibiotics SMX and TMP are also not efficiently removed by WWTPs (Göbel et al., 2005) and different processes (e.g. UVA/LED/TiO_2 photocatalysis, use of the green algae *Nannochloris* sp.) only partially remove SMX and TMP (Bai & Acharya, 2016; Cai & Hu, 2017).

Consequently, in order to reduce the constant entry of pharmaceuticals in the environment, different strategies have to be taken into consideration. A possible alternative might be the implementation of a pollution prevention approach, aiming in better controlling the magnitude of consumption and consequent excretion by reducing doses and concomitantly prescribing those medications that would have the lesser impact on the environment (Daughton, 2014). Different approaches have been proposed such as the use of pharmEcokinetics factors (Deblonde & Hartemann, 2013) or the eco-directed sustainable prescribing (EDSP) (Daughton, 2014), that, if implemented together could effectively reduce the presence of certain pharmaceuticals in the environment, without jeopardizing patients' health.

Data regarding environmental fate and toxicity of pharmaceuticals in the aquatic environment could also serve the purpose of comprising together information to implement a pollution prevention approach that could be used by the healthcare industry to minimise its ecological footprint.

6.6 Conclusions

In conclusion, the current research has highlighted how behavioural endpoints (e.g. feeding and swimming velocity) are a useful and sensitive tool in aquatic ecotoxicology to understand the potential sublethal effects that pharmaceuticals might pose to non-target organisms. Indeed, behaviour is considered to be the bridge connecting alterations in environmental conditions and changes in the physiological state of the organism (Pyle & Ford, 2017).

Three different classes of pharmaceuticals (e.g. antidiabetic, antidepressant and antibiotics) have been tested on *G. pulex* and several changes in behaviour were measured (e.g. feeding activity, swimming velocity and movement). Alteration in behavioural can have detrimental effects on essential mechanism (e.g. growth, reproduction and survival) and this could have profound repercussions on the entire population. Moreover, *G. pulex* plays a key role in riverine environments and consequently, shifts in *G. pulex* populations can have negative consequences on the entire ecosystem.

The current study also emphasised the necessity of standardising behavioural analyses, as there are still considerable differences in the adopted methodologies, making it more difficult for ecotoxicologists to compare different studies and for policy makers to take advantage of these non-destructive and cost-effective protocol by integrating them in environmental risk assessments and in monitoring programs.

Lastly, studying the effects of different pharmaceuticals is important, not only to understand the impact that these compounds have on the environment, but also to improve current legislations and implement a common line of discussion between all the parties involved (e.g. consumers, manufacturers, distributers, prescribers, environmental agencies etc), so that the classic end-pipe pollution control can evolve into a prime source prevention approach.

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Chapter 8: Appendices

Appendix A

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Feeding Behavioural Studies with Freshwater *Gammarus* spp.: The Importance of a Standardised Methodology

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Contents

Abbreviation

AFDW Ash-free dry weight

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1 Introduction

Freshwater Gammarids are common leaf-shredding detritivores, and they usually feed on naturally conditioned organic material, in other words leaf litter that is characterised by an increased palatability, due to the action and presence of micro-organisms (Chaumot et al. 2015; Cummins 1974: Maltby et al. 2002). *Gammarus* spp. are biologically omnivorous organisms, so they are involved in shredding leaf litter and are also prone to cannibalism, predation behaviour (Kelly et al. 2002) and coprophagy when juveniles (McCahon and Pascoe 1988). *Gammarus* spp. is a keystone species (Woodward et al. 2008), and it plays an important role in the decomposition of organic matter (Alonso et al. 2009; Bundschuh et al. 2013) and is also a noteworthy prey for fish and birds (Andrén and Eriksson Wiklund 2013; Blarer and Burkhardt-Holm 2016). Gammarids are considered to be fairly sensitive to different contaminants (Ashauer et al. 2010; Bloor et al. 2005; Felten et al. 2008a; Lahive et al. 2015; Kunz et al. 2010); in fact Amphipods have been reported to be one of the most sensitive orders to metals and organic compounds (Wogram and Liess 2001), which makes them representative test organisms for ecotoxicological studies and valid sentinel species for assessing water quality status (Garcia-Galan et al. 2017).

Since Gammarids play an important role in the breakdown of organic matter in freshwater environments, it is understandable that their feeding behaviour is often used as a sublethal endpoint, to investigate water quality status and the effects of different contaminant types (Crane and Maltby 1991). Gammarid feeding activity could be altered by the presence of contaminants in the water, which could potentially alter their food source, influence the organism's biological function and cause abnormal behavioural responses. These types of feeding investigation have been carried out as in situ (i.e. directly in the environment) and ex situ (i.e. in the laboratory) studies (Bundschuh et al. 2011b; Dedourge-Geffard et al. 2009; Maltby et al. 2002; Zubrod et al. 2015). It has been demonstrated that feeding assays using Gammarids are representative of natural leaf decomposition in the environment (Maltby et al. 2002) and could be used to assess the effects of chemical contaminants and also understand the consequences of new-generation contaminants, such as plastic debris in freshwater environments (Blarer and Burkhardt-Holm 2016; Weber et al. 2018). Even though feeding behaviour studies have been carried out for almost half a century, there is a lack of standardisation for both ex situ and in situ methods. Without standardisation, there is a risk that the effects of a test substance could be under- or overestimated during in situ and ex situ approaches, which could reduce their usefulness in environmental biomonitoring programs. This paper aims to review the literature on feeding as an endpoint for amphipod ecotoxicology, by highlighting disparities in the published methodologies, and to help develop standardised protocols. Peer-reviewed literature was accessed through search engines, databases and library archives. In general, most feeding studies have reported four main stages: (1) acclimation period, (2) food preparation, (3) exposure and (4) end of the experiment and feeding rate calculation. The aforementioned four

stages have been reviewed separately, and the variability of the published methodologies has been considered, in order to draw attention to the current discrepancies in the literature.

2 Acclimation Conditions

The first stage of an experiment (both in situ and ex situ) is the acclimation period that should be used to acclimate the organisms to the experimental conditions. However, the acclimation conditions are not always fully disclosed, and when they are, they sometimes contradict the experimental conditions. The reproducibility of an experiment is also highly dependent on many abiotic and biotic factors, which are rarely taken into consideration for Gammarid feeding studies (Coulaud et al. 2011). In the following sections, different variables (duration, temperature, light/dark cycles, type of water and organisms) that could impact the outcome of an experiment have been reviewed separately and summarised in Table 1, in order to emphasise the full range of variability within the literature. In some studies, Gammarids are sourced from laboratory breeding programs (e.g. Blockwell et al. 1996; Bloor and Banks 2006a, b; McCahon and Pascoe 1988).

2.1 Duration

Acclimation periods vary depending on the study (see Table 1), for example, Agatz et al. (2014) kept specimens of *Gammarus pulex* in the laboratory for 3 days prior to the start of the experiment, whereas another study left *Gammarus fossarum* organisms to acclimate for 21 days (Garcia-Galan et al. 2017). Typically the acclimation period used for Gammarids appears to be between 5 and 7 days, but some studies have selected longer intervals up to 35 days (see Table 1). Agatz and Brown (2014) stated that a 1-day acclimation period helped to reduce the variability of their results by just 1.6%, suggesting that a longer acclimation period could potentially have an even greater impact on reducing the intraspecific variability and consequently strengthen the statistics. Although experimental controls are incorporated into the majority of experimental designs, it becomes difficult to compare published peer-reviewed research when the test organisms have experienced anything between 3 and 35 days acclimation to laboratory conditions (Agatz et al. 2014; Garcia-Galan et al. 2017) (see Table 1), even more so when the organisms are used as water quality biomonitors for in situ experiments (see Table 1).

Table 1 Existing differences in the literature regarding *Gammarus* spp. acclimation conditions

Test organism	Age/sex/size organism	Duration	Temperature	Light/dark cycle	Type of water Aeration pH	Type of study	References
Gammarus fossarum	Free from parasites No gravid females	7 days	16°C	12:12	Mixed water Aerated	Feeding and assimilation study	Blarer and Burkhardt-Holm (2016)
Gammarus fossarum	Adults with a cephalothorax length between 1.2 and 1.6 mm	7 days	15°C		River water	Feeding preferences study	Bundschuh et al. (2009)
Gammarus fossarum	Conducted as described by Bundschuh et al. (2009)					Feeding rate study	Bundschuh et al. (2011a)
Gammarus fossarum	Free from parasites Adults with a cephalothorax length between 1.2 and 1.6 mm	7 days	15°C		River water	Feeding rate study	Bundschuh et al. (2011b)
Gammarus fossarum	Free from parasites No gravid females Adults with a cephalothorax length between 1.2 and 1.6 mm	7 days	15°C		River water	Feeding rate study	Bundschuh et al. (2013)
Gammarus fossarum	Adults with a cephalothorax length between 1.2 and 1.6 mm	7 days	15°C	Total darkness	River and tap water mixture	Feeding behavioural study	Bundschuh et al. (2017)
Gammarus fossarum	Juveniles and adult males	15 days	12°C	10:14	Groundwater mixed with osmosed water Aerated	Ex situ and in situ feeding assay	Coulaud et al. (2011)
Gammarus fossarum	Dry mass = 6.8 ± 0.7 mg	7 days	10°C			Decomposition and feeding rate study	Danger et al. (2012)

Gammarus fossarum		20–25 days	12°C	10:14	Drilled groundwater Aerated	In situ feeding experiment	Dedourge-Geffard et al. (2009)
Gammarus fossarum	Adult males	21 days	12°C	10:14	Groundwater Aerated	Bioaccumulation study	Garcia-Galan et al. (2017)
Gammarus fossarum	Adult females	30–35 days	12°C	16:08	Drilled groundwater Aerated	Reproductive cycle and feeding study	Geffard et al. (2010)
Gammarus fossarum	Adult males with diameter from 1.6 to 2.0 mm	7 days	16°C	Total darkness	SAM-5S medium	Feeding rate study	Newton et al. (2018)
Gammarus fossarum		10 days	12°C	8:16	Drilled groundwater Aerated	Feeding behaviour and biomarkers analysis	Xuereb et al. (2009)
Gammarus fossarum	Free from parasites Adults with a cephalothorax length between 1.2 and 1.6 mm	7 days	15°C		River water Aerated	Feeding, accumulation and growth study	Zubrod et al. (2010)
Gammarus fossarum	Free from parasites Adult males (6–8 mm)	7 days	20°C	Total darkness	Aerated medium	Feeding and survival study	Zubrod et al. (2014)
Gammarus fossarum	Free from parasites Adult males (6–8 mm)	7 days	16°C	Total darkness	SAM-5S medium Aerated	Toxicity and feeding study	Zubrod et al. (2015)
Gammarus fossarum	Free from parasites Different sizes	3 days	16°C		SAM-5S medium Aerated	Feeding behavioural and physiological responses	Zubrod et al. (2017)
Gammarus pseudolimnaeus	Juveniles and adults					Feeding behavioural study	Bärlocher and Kendrick (1973b)
Gammarus pulex	Free from parasites Dry body mass 3.8–15 mg	3 days	13°C	12:12	Artificial pond water	Feeding rate study	Agatz et al. (2014)

(continued)

Table 1 (continued)

Test organism	Age/sex/size organism	Duration	Temperature	Light/dark cycle	Type of water Aeration pH	Type of study	References
Gammarus pulex	Organisms with parasites Both sexes Juveniles and adults	1 day	13°C	12:12	Artificial pond water Aerated pH = 7.4–7.9	Feeding rate studies	Agatz and Brown (2014)
Gammarus pulex	Free from parasites Adults (mean size 9.7 ± 1.4 mm) No gravid females	1. 4 days 2. 4 days	1. 15°C 2. 20°C		River water Artificial water Aerated	Feeding rate study with the Multispecies Freshwater Biomonitor	Alonso et al. (2009)
Gammarus pulex	3–7 mm		13°C	12:12	Dechlorinated tap water pH = 7.7	Feeding behavioural study	Blockwell et al. (1998)
Gammarus pulex	Adult males	7 days	15°C	12:12		In situ feeding assay	Crane and Maltby (1991)
Gammarus pulex	Males with first thoracic segment of 0.7–1.2 mm in size	7 days	15°C	12:12	River water	Feeding behavioural study	De Castro-Català et al. (2017)
Gammarus pulex	Adults (7–9 mm)	10 days	12°C		Well water pH = 7.19 ± 0.02	Physiological and behavioural responses	Felten et al. (2008a)
Gammarus pulex	Free from parasites Adult males (dry weight 6.5–12.0 mg)		15°C	12:12	Artificial pond water	In situ and laboratory feeding studies	Forrow and Maltby (2000)
Gammarus pulex	Adults (dry weight 8–10 mg)					Feeding behavioural study	Graça et al. (1993a)
Gammarus pulex	Adults (9–10 mm) Juveniles (2.5–3.5 mm)		15°C	12:12	Artificial pond water	Feeding behavioural study	Graça et al. (1993b)
Gammarus pulex	Adults		13°C			Feeding behavioural study	Hahn and Schulz (2007)

Gammarus pulex	Wet weight = 1.5–2.5 mg	10 days			Dechlorinated city tap water	Growth and feeding rate study	Hargeby and Petersen (1988)
Gammarus pulex	Both sexes		14°C	12:12	River water	Energetic state study	Iltis et al. (2017)
Gammarus pulex	Males (13–16 mm)		12°C	14:10	Aerated	Predation behaviour study	Kelly et al. (2002)
Gammarus pulex	Adults	7–14 days	19–22°C		Dechlorinated tap water Aerated pH = 8.28 ± 0.06	Feeding and bioaccumulation study	Lahive et al. (2015)
Gammarus pulex	Adult males (mean dry weight = 8.24 mg)	5–10 days	15°C	12:12	Artificial pond water	In situ feeding assay	Maltby et al. (2002)
Gammarus pulex	Adult males (dry weight = 7–10 mg)	7 days	15°C	12:12	Artificial pond water	Scope for growth assay	Naylor et al. (1989)
Gammarus pulex		1 day	14°C	16:8		Feeding behavioural study	Taylor et al. (1993)
Gammarus pulex	Adults Juveniles	7 days	16°C	16:8	ISO medium Aerated	Feeding activity and physiological responses	Weber et al. (2018)
Gammarus roeselii	Both sexes		15°C	12:12	Lake water	Feeding, assimilation and growth study	Gergs and Rothhaupt (2008)
Gammarus spp.		7 days			River water Aerated	Selective feeding study	Arsuffi and Suberkropp (1989)
Gammarus spp.		5 days	10°C		River water pH = 7.2	Physiological and behavioural responses	Maul et al. (2006)

2.2 Temperature

During the acclimation period, organisms need to be kept at a constant temperature and with a precise light/dark cycle. Gammarids from temperate countries are usually maintained at a temperature between 10 and 22°C (see Table 1). The temperature adopted in an experimental design is often selected to reproduce seasonal conditions, but unfortunately the literature does not always specify the selection criteria. Temperature can have a significant impact on Gammarids and on amphipods in general (Labaude et al. 2017). Foucreau et al. (2014) discovered that temperatures higher than 15°C altered various physiological parameters in *Gammarus pulex* populations in North France. Southern specimens consumed more oxygen at higher temperatures and had a higher glycogen content, which means they have a higher energy supply. Cold-acclimated organisms consumed more energy and oxygen when they are exposed to higher temperatures, and they presented a lower heat tolerance (Semsar-kazerouni and Verberk 2018). Interestingly, Alonso et al. (2009) acclimated their organisms at 15°C for 4 days, after which time the organisms were transferred to a 20°C room to acclimate for a further 4 days. Moving organisms from a low to a high temperature could have potentially affected the experimental results (Alonso et al. 2009). Furthermore, temperature plays an important role in the immune system of crustaceans (Le Moullac and Haffner 2000). Therefore, it is difficult to compare studies where the test animals have been acclimated at different temperatures, as this could have influenced their energy stores or their immune systems, for example. These differences could also be reflected in the organisms' behavioural reactions, which could be incorrectly interpreted as a result of exposure to specific contaminants. In fact, both Nilsson (1974) and Coulaud et al. (2011) reported an increased feeding rate with an increased temperature. The extent of the feeding rate increase was also dependent on leaf species (i.e. *Alnus glutinosa* or *Fagus sylvatica*) (Nilsson 1974). Acclimation temperature plays an even greater role in in situ experiments where the chosen temperature should be as close as possible to real-life environmental conditions. Interestingly, Coulaud et al. (2011) linked temperature and feeding rate through a linear regression, in order to better understand the impact of temperature on the Gammarids feeding. It was found that a small increase in mean temperature (from 12 to 13°C) could enhance the feeding rate by 7.3%.

2.3 Light and Dark Cycles

The same principle could be applied to the different light/dark cycles used during the acclimation period. The most commonly adopted light/dark cycle is 12:12 h (see Table 1) that reflects typical equinox conditions. However, some studies acclimate their organisms in total darkness, and in other studies, the adopted cycle is not specified (see Table 1). Sometimes a seasonal cycle is selected, in order to replicate the time of year when the organisms are collected from the wild, such as summer

with a light/dark cycle of 16:8 h (Weber et al. 2018) or autumn with a cycle of 10:14 h (Garcia-Galan et al. 2017) (see Table 1). Adopting different light/dark cycles could make the comparison between studies challenging, since light could influence the organisms' physiological processes and behaviour (Perrot-Minnot et al. 2013).

2.4 Media Selection

The type of media selected for an experiment is another factor that could have an impact on the outcome of a study. Some researchers prefer to use an artificial medium (see Table 1) that guarantees standardisation (Agatz et al. 2014; Maltby et al. 2002), and in other studies, river water is sometimes used as a medium. However, river water might be contaminated, and this could therefore interfere with the organisms' cleansing process during their acclimation period, which makes it a peculiar choice of test media. Numerous studies have also used river water or a mixture (Alonso et al. 2009; Blarer and Burkhardt-Holm 2016; Bundschuh et al. 2009, 2017; De Castro-Català et al. 2017; Dedourge-Geffard et al. 2009; Gergs and Rothhaupt 2008; Iltis et al. 2017; Maul et al. 2006; Zubrod et al. 2015) (see Table 1). For example, Bundschuh et al. (2017) combined river water with tap water, which also has limitations as the tap water could be contaminated (Magi et al. 2018). Potentially, any type of water could be contaminated, which is why the authors recommend that researchers should report the chemical breakdown (i.e. presence of contaminants) of their chosen water media along with their study findings so that any contamination is transparent.

Gammarus pulex allocates up to 11% of its energy supply to osmotic regulation (Sutcliffe 1984), and Gammarids have been proven to be acid-sensitive (*Gammarus fossarum*; Felten and Guerold 2001; *Gammarus pulex*, Sutcliffe and Carrick 1973). In fact, acidic conditions induce a range of physiological and behavioural alterations, such as a reduction in the ventilation activity of *Gammarus pulex* (Felten et al. 2008b). These findings highlight the importance of measuring pH, as a shift in pH might influence the outcome of an experiment and prevent comparisons between studies. pH is rarely reported and presumably not measured in the environment during the collection process, the acclimation period or the experiment. Along with the chemical parameters of the acclimation media, the authors also recommend that pH is another factor that should be measured during the acclimation period, to ensure that accurate baseline data is recorded.

2.5 Characteristics of the Test Organism

Another important factor that plays a fundamental role in the reproducibility of a feeding experiment is the organism itself. Organisms of different age and sex may behave or respond differently to contaminants. For example, juveniles are more

sensitive to contaminants than adult organisms (Adam et al. 2010), and their feeding rate varies over time, making them more suitable for short-term feeding studies (Agatz and Brown 2014). Agatz and Brown (2014) and Nilsson (1974) identified that smaller specimens of *Gammarus pulex* have a higher feeding rate but higher variability over time, in comparison to larger organisms.

However, other studies have reported that the feeding rate increases with organism size (Coulaud et al. 2011), but the adoption of different units of measurement and a small size range might be contributing factors for those findings. It has been suggested that using organisms with a specific body mass (given in dry weight) could reduce experimental variability. For feeding studies, up to a 57% reduction in variability has been documented for specific body mass studies compared to mixed body mass studies (Agatz and Brown 2014). There is also a recommendation that body length should be used as an indicator of dry weight and the correlation for organisms between 2 and 16 mm (Graça et al. 1993b).

Alternatively, organisms might be divided into size groupings by applying passive underwater separation techniques (Bundschuh et al. 2009, 2017; Zubrod et al. 2017), by measuring the dorsal length of the Gammarids' first thoracic segment after the organisms are photographed (De Castro-Català et al. 2017), by considering their wet weight (Blockwell et al. 1996; Danger et al. 2012; Weber et al. 2018) or by using their dry weight at the end of an experiment (Agatz et al. 2014). There is no agreed standard method on how to separate or select specimens of Gammarids for this experimental technique, but the chosen method will ultimately determine the unit of measurement for calculating the feeding rate, e.g. if wet weight is used, the unit of measurement will be wet weight. The use of either dry or wet weight seems straightforward, but it is only an estimate, and it lacks accuracy, as the dry weight range is only known at the end of the study. Furthermore, wet weight does not provide an accurate measurement due to the unknown volume of liquid in each sample. Blotting the sample dry before weighing could help to remove a proportion of the moisture, but it could potentially stress the organisms and consequently affect the results; therefore, the authors recommend the use of dorsal length as the authors believe it to be a more accurate way to measure the organisms. In in situ experiments, the organisms are often divided by size before the start, but the weight is not taken into consideration. This means that the amount of consumed food is usually related to the number of living organisms at the end of the experiment (e.g. Coulaud et al. 2011; Dedourge-Geffard et al. 2009) (see Table 3).

Same-sex tests with organisms (female-only, Geffard et al. 2010, or male-only, Crane and Maltby 1991; De Castro-Català et al. 2017; Forrow and Maltby 2000; Kelly et al. 2002; Maltby et al. 2002; Naylor et al. 1989; Zubrod et al. 2015) (see Table 1) of a specific size are often undertaken, although sex is not always specified, which leads to female and male organisms being used indiscriminately (Agatz et al. 2014; Agatz and Brown 2014; Alonso et al. 2009; Arsuffi and Suberkropp 1989; Bärlocher and Kendrick 1973a; Blarer and Burkhardt-Holm 2016; Blockwell et al. 1998; Bundschuh et al. 2009, 2011b, 2013, 2017; Dedourge-Geffard et al. 2009;

Gergs and Rothhaupt 2008; Graça et al. 1993a, b; Hahn and Schulz 2007; Lahive et al. 2015; Taylor et al. 1993; Weber et al. 2018; Xuereb et al. 2009; Zubrod et al. 2017) (see Table 1).

As a rule, and not only in feeding studies, gravid females and organisms affected by the acanthocephalan parasite are usually excluded from experiments (Agatz et al. 2014; Alonso et al. 2009; Blarer and Burkhardt-Holm 2016; Bundschuh et al. 2011b, 2013; Forrow and Maltby 2000; Zubrod et al. 2015, 2017) unless they are specifically chosen for the purpose of the study (Agatz and Brown 2014; Pascoe et al. 1995). Alonso et al. (2009) developed a feeding study using the Multispecies Freshwater Biomonitor, and neither length nor sex influenced the feeding activity of either sex of *Gammarus pulex*. However, it is debatable whether these results might only be applicable to the type of contaminant used in the investigation, as some contaminants might affect male and female Gammarid feeding behaviour in different ways.

3 Food Preparation

The food source selected for an experiment using Gammarids is important, but especially so for feeding studies, both in the acclimation period and in the experiment itself. Gammarids are shredder detritivores, and they usually feed on conditioned organic material, in other words material that has been colonised by microorganisms, such as leaf litter. In the natural environment, freshly abscised leaves are colonised by fungi and then by bacteria (Baldy et al. 1995), which facilitate the decomposition process and transform the material, making it more palatable and accessible to the organisms (Bärlocher and Kendrick 1975; Cummins 1974; Gessner et al. 1999).

Gammarus spp. have displayed selective behaviour towards leaf species and their conditioning level (Agatz and Brown 2014; Graça et al. 1993a, b, 2001) and the type of fungi (Arsuffi and Suberkropp 1989). Interestingly, Graça et al. (2001) compared food preferences of shredders from temperate (*Gammarus pulex* and *Sericostoma vittatum*) and tropical (*Nectopsyche argentata* and *Phylloicus priapulus*) streams. When provided with conditioned and/or unconditioned leaves from either a temperate (*Alnus glutinosa*) or tropical (*Hura crepitans*) country, *Gammarus pulex* showed a significant preference for the conditioned leaves compared to unconditioned leaves of the same species. Leaves are characterised by different hardness, texture and more importantly by dissimilar C:N ratios, which means the various leaf species provide the organisms with differing energy supplies. A lower C:N ratio signifies a better quality food, and conditioned material is usually characterised by a lower C:N ratio compared to unconditioned material (Graça et al. 1993b). Some species such as alder (*Alnus* spp.) are characterised by a lower C:N ratio and higher palatability compared to others, such as horse chestnut (*Aesculus* spp.) (Agatz and Brown 2014), which could lead to the organisms growing larger (Bärlocher and Kendrick 1973b).

In feeding assays, there are several options when considering a food source. The most common choice is to provide the Gammarids with conditioned organic material. Depending on the study, the adopted leaf species may be different. The most commonly used leaves are alder (*Alnus* spp.), elm (*Ulmus* spp.), horse chestnut (*Aesculus* spp.), maple (*Acer* spp.), poplar (*Populus* spp.) and oak (*Quercus* spp.) (see Table 2). In some cases, the Gammarids' diet is enriched with *Tubifex* worms (Coulaud et al. 2011; Dedourge-Geffard et al. 2009; Geffard et al. 2010; Xuereb et al. 2009). Occasionally, they are provided with other types of food, such as alimentary chips (Novo Crabs®, JBL GmbH & Co., Germany) (Foucreau et al. 2014), Chironomidae (Gergs and Rothhaupt 2008), *Artemia salina*'s eggs (Blockwell et al. 1998; Pascoe et al. 1995; Taylor et al. 1993), industrial shrimp food (Henry et al. 2017), fish food (Semsar-kazerouni and Verberk 2018) or ground and tropical fish food mix (Blockwell et al. 1996).

During the acclimation period, organisms are normally fed ad libitum with pre-prepared conditioned leaves (Blarer and Burkhardt-Holm 2016; Blockwell et al. 1998; Bloor 2010; Bundschuh et al. 2011b; Crane and Maltby 1991; Dedourge-Geffard et al. 2009; Geffard et al. 2010; Naylor et al. 1989; Newton et al. 2018; Xuereb et al. 2009; Zubrod et al. 2015). The conditioning process can vary, and the differences between the techniques can be found in Table 2.

In behavioural studies, food is supplied to the organism during the testing regime and is usually the same food type as provided during the acclimation period. The type of food used in a study could influence the feeding activity, especially if the organisms are fed on leaves that are not palatable or with leaves that have dissimilar energy budgets (e.g. Agatz and Brown 2014).

Sometimes leaves are collected at the beginning or during fall, specifically handpicked senescent *Alnus glutinosa* leaves that are not decomposed (Bundschuh et al. 2009, 2017), whereas in other studies, the leaves are specifically collected after they had abscised (Hargeby and Petersen 1988). After collection the leaves are either used straight away or stored for later use (see Table 2). Storage methods vary throughout the literature, for example, Bundschuh et al. (2009, 2011a, b, 2013, 2017) froze their leaves at −20°C, but this methodology ultimately alters the structure of the leaves (Burke et al. 1976). More commonly, the leaves are dried at room temperature and stored in the dark until needed (e.g. Naylor et al. 1989) (see Table 2). However, Gessner et al. (1999) highlighted that drying leaves in an oven or at room temperature ultimately ruins the leaf tissue. In the natural environment, leaves usually reach water bodies soon after abscission (Fisher 1977). Consequently, storing leaves for later use does not mimic the natural chain of events, and storing will ultimately disrupt their structure. Gessner and Schwoerbel (1989) demonstrated that freezing or drying leaves increases mass loss in the first few days when in water, and this accelerates the conditioning process, which is usually statistically delayed in fresh leaves (Bärlocher 1992).

The conditioning process involves soaking the leaves in water and mixing them with an unknown fungi species (Nilsson 1974) or by inoculating the leaves with a specific fungi species (Naylor et al. 1989). In the first instance, river water might be used in the laboratory to condition the leaves, and it is usually inoculated with

Table 2 Existing differences in the literature regarding the conditioning process

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during acclimation	Type of study	Reference
Acer saccharum *Ulmus* spp.	Cut in discs of 9 mm in diameter, leached in tap water for 4 days at 12°C, and dried at 40°C for 2 days Stored at room temperature	*Experiment food:* nutrient-enriched river water	*Alternaria* sp. *Fusarium* sp. *Cladosporium* sp. *Humicola grisea* *Aspergillus niger* *Tricladium angulatum* *Tetracladium marchalianum* *Anguillospora longissima* *Clavariopsis aquatica* *Flagellospora curvula*				Feeding study	Bärlocher and Kendrick (1973b)
Acer saccharum	Air-dried and stored in plastic bags until use	*Experiment food*: river water		4 days	After being cut in leaf discs of 1.1 cm in diameter and being dried for 3 days at 60°C		Physiological and behavioural responses	Maul et al. (2006)
Aesculus hippocastanum	*Acclimation food:* stored in tap water for 3 months *Experiment food:* dried at room temperature	*Acclimation food:* tap water *Experiment food:* enriched water	*Cladosporium* spp.	10 days	*Experiment food:* before re-drying at 60°C	Ad libitum	Feeding study	Agatz et al. (2014)
1. *Aesculus hippocastanum* 2. *Alnus glutinosa*	*Acclimation food:* whole horse chestnut leaves stored in tap water *Experiment food*:	*Acclimation food:* tap water *Experiment food:* 1. Enriched water or tap water	*Acclimation food:* *Cladosporium* spp. *Experiment food:*	*Acclimation food:* 3 months *Experiment food:*	*Experiment food:* 2. After being cut in discs of 2.0 cm in diameter, but before re-drying at 60°C		Feeding study	Agatz and Brown (2014)

(continued)

Table 2 (continued)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during acclimation	Type of study	Reference
	1. Air-dried in the dark at room temperature (20°C)	2. Nutrient medium	1. *Cladosporium* spp.	1. 2 weeks or 3 months 2. 10 days				
Aesculus hippocastanum		*Acclimation food:* conditioned in organically enriched dechlorinated water (Bird and Kaushik 1985)		10 days		Ad libitum	Feeding study	Blockwell et al. (1998)
Aesculus hippocastanum		Organically enriched water, following method of Bird and Kaushik (1985)		10 days			Feeding study	Taylor et al. (1993)
Alnus glutinosa	Air-dried for 1 h and then stored at −20°C	*Acclimation food:* decaying leaves collected in a pond *Experiment food:* cut in leaf discs of 2 cm in diameter and conditioned directly in the river		21 days	After being cut in leaf discs of 2 cm in diameter, but before been dried at 60°C for 24 h Soaked for 24 h in water before being used in the experiment	*Acclimation food*: ad libitum	Feeding and assimilation study	Blarer and Burkhardt-Holm (2016)
Alnus glutinosa	Froze at −20°C until use	*Experiment food:* soaked in tap water for 24 h and then conditioned in a nutrient medium with added leaf litter from the river		From 19 to 22 days	After being cut in leaf discs of 1.5 cm in diameter and been dried at 60°C for 24 h		Feeding preferences study	Bundschuh et al. (2009)

Alnus glutinosa	Frozen at −20°C and stored until use. Following the method described by Bundschuh et al. (2009)	*Acclimation food:* preconditioned leaves *Experiment food:* conditioning medium (Dang et al. 2005) inoculated with leaves previously conditioned directly in the river		12 days	After being cut in leaf discs of 1.6 cm in diameter and dried		Feeding preferences study	Bundschuh et al. (2011a)
Alnus glutinosa	Froze at −20°C until use	*Acclimation food:* preconditioned alder leaves *Experiment food:* conditioned in a nutrient medium with added leaves that were previously conditioned directly in the river		10 days	After being cut in leaf discs of 2 cm in diameter, but before been dried at 60°C for 24 h	Preconditioned alder leaves fed ad libitum	Feeding study	Bundschuh et al. (2011b)
Alnus glutinosa	Froze at −20°C until use	*Acclimation food:* preconditioned alder leaves *Experiment food:* conditioned in a nutrient medium with added leaves that were previously conditioned directly in the river		10 days	After being cut in leaf discs of 2 cm in diameter, but before been dried at 60°C for 24 h		Feeding study	Bundschuh et al. (2013)

(continued)

Table 2 (continued)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during acclimation	Type of study	Reference
Alnus glutinosa	Froze at −20°C until use	*Acclimation food:* preconditioned Alder leaves *Experiment food:* conditioned in a mixture of tap water and stream water with preconditioned leaves coming from the river		18 days	Before being cut in leaf discs of 2 cm in diameter and been dried at 60°C for 24 h Soaked for 24 h in tap water before being used in the experiment		Feeding study	Bundschuh et al. (2017)
Alnus glutinosa		*Acclimation and experiment food:* groundwater Freeze-dried *Tubifex* worms added to the food twice a week		6 days		Ad libitum	Laboratory and in situ feeding study	Coulaud et al. (2011)
Alnus glutinosa			*Cladosporium*			Ad libitum	In situ feeding study	Crane and Maltby (1991)
Alnus glutinosa		*Experiment food:* conditioned in a river water		14 days			Feeding study	De Castro-Català et al. (2017)
Alnus glutinosa		*Acclimation and experiment food:* conditioned in water Freeze-dried *Tubifex* worms added to the food twice a week		6 days		*Acclimation food*: *Ad* libitum	In situ feeding study	Dedourge-Geffard et al. (2009)

Alnus glutinosa	Air-dried and stored at room temperature until use	*Acclimation food:* fungally conditioned leaves *Experiment food:* two different types depending on the experiment 1. Leaves were naturally conditioned in the river 2. Method described by Naylor et al. (1989)	2. *Cladosporium*		Before being cut in leaf discs of 1 cm in diameter and been blotted dry and weighed Used immediately afterwards		In situ and laboratory feeding studies	Forrow and Maltby (2000)
Alnus glutinosa		*Acclimation food:* conditioned in water Freeze-dried *Tubifex* worms added to the food twice a week		6 days		*Acclimation food*: ad libitum	Reproductive cycle and feeding study	Geffard et al. (2010)
Alnus glutinosa		*Experiment food:* naturally conditioned in the lake		21 days			Feeding, assimilation and growth study	Gergs and Rothhaupt (2008)
Alnus glutinosa *Hura crepitans*	Air-dried and stored until use	*Experiment food*: different types. Depending on the experiment 1. Conditioned directly into the river 2. Leaves soaked in tap water		1. 14 days 2. 24 h	Before being cut in leaf discs of 1.4 cm in diameter		Feeding study	Graça et al. (2001)
Alnus glutinosa	Frozen at −20°C until use	*Experiment food:* river water		14 days	After being cut in leaf discs of 2 cm in diameter		Feeding study	Hahn and Schulz (2007)
Alnus glutinosa		*Acclimation and experiment food:* method described by Naylor et al. (1989)					In situ feeding study	Maltby et al. (2002)

(continued)

Table 2 (continued)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during acclimation	Type of study	Reference
Alnus glutinosa	Dried and stored	*Acclimation and experiment food:* enriched water	*Cladosporium* spp.	10 days	After being rehydrated, cut in leaf discs of 1.6 cm in diameter and autoclaved, but before being dried for 2 days at 60°C Rehydrated again before being fed	*Acclimation food*: ad libitum	Scope for growth assay	Naylor et al. (1989)
Alnus glutinosa	Froze at −20°C	*Acclimation food:* preconditioned leaves *Experiment food:* 1. Conditioning medium as Dang et al. (2005) inoculated with alder leaves conditioned in the river for 14 days 2. Conditioning medium		1. 13 days 2. 14 days	1. After being cut in leaf discs of 1.6 cm in diameter, being froze for 24 and subsequently weighed Unconditioned leaves either submerged for 2 min or 48 h in SAM-5S medium 2. Before being cut in leaf discs of 2.0 cm in diameter and being directly fed to the organisms	*Acclimation food:* ad libitum	Feeding study	Newton et al. (2018)
Alnus glutinosa Fagus sylvatica	Dried at 20°C	*Experiment food:* river water		10 days			Feeding, assimilation and respiration study	Nilsson (1974)
Alnus glutinosa		*Acclimation and experiment food:* conditioned in water Freeze-dried *Tubifex* worms added to the food twice a week		6 days		*Acclimation food*: ad libitum	Feeding behaviour and biomarkers analysis	Xuereb et al. (2009)

Alnus glutinosa	Frozen at −20°C and stored until use. Following the method described by Bundschuh et al. (2011b)	*Acclimation food:* preconditioned leaves *Experiment food:* conditioning medium (Dang et al. 2005) inoculated with leaves previously conditioned directly in the river		10 days	After being cut in leaf discs of 2.0 cm in diameter, but before being dried at 60°C for 24 h and weighed Soaked for 24 h in tap water before being used in the experiment	*Acclimation food*: ad libitum	Feeding, accumulation and growth study	Zubrod et al. (2010)
Alnus glutinosa	Frozen at −20°C and stored until use. Following the method described by Zubrod et al. (2010)	*Acclimation food*: preconditioned leaves *Experiment food*: conditioning medium (Dang et al. 2005) inoculated with leaves previously conditioned directly in the river		10 days	After being cut in leaf discs of 2.0 cm in diameter, but before being dried at 60°C for 24 h and weighed. Soaked for 48 h in tap water before being used in the experiment	*Acclimation food*: ad libitum	Feeding an survival study	Zubrod et al. (2014)
Alnus glutinosa	Frozen at −20°C and stored until use	Conditioned in medium with leaves that were previously conditioned directly in the river for 14 days		12 days		*Acclimation food*: ad libitum	Toxicity and feeding study	Zubrod et al. (2015)
Alnus glutinosa	Frozen at −20°C and stored until use	Conditioned in stream water with leaves that were previously conditioned directly in the river for 14 days and for 14 days in the lab		13 days			Feeding behaviour and physiological responses	Zubrod et al. (2017)
Alnus spp.	Air-dried and stored	River water with detritus		At least 10 days	After being air-dried	Ad libitum	Laboratory breeding program	Bloor (2010)

(continued)

Table 2 (continued)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during acclimation	Type of study	Reference
Fraxinus pennsylvanica *Acer saccharum* *Quercus velutina*	Cut in discs of 1–2 cm in diameter, leached in tap water for 4 days at 12°C, and dried at 40°C for 2 days Stored at room temperature in polyethylene bags		*Alternaria* spp. *Fusarium* spp. *Cladosporium* spp. *Aspergillus niger* *Humicola grisea* *Tricladium angulatum* *Tetracladium marchalianum* *Anguillospora longissima* *Clavariopsis aquatica* *Flagellospora curvula*	14 days	After drying			Bärlocher and Kendrick (1973a)
Populus sp.		*Acclimation food:* conditioned naturally in the river *Experiment food*: mixture of stream water and Dutch Standard Water (DSW)		*Acclimation food*: *Experiment food:* 4 days			Feeding rate study with the Multispecies Freshwater Biomonitor	Alonso et al. (2009)
Populus tremuloides		*Experiment food:* incubation medium Stream water	*Flagellospora curvula* *Alatospora acuminata* *Clavariopsis aquatic*	Either 10 or 15 days depending on the fungi and then 72 h in stream water	*Experiment food*: before drying at 45°C		Selective feeding study	Arsuffi and Suberkropp (1989)

			Tetracladium marchalianum *Lemonniera aquatica* *Heliscus lugdunensis* *Articulospora inflata* *Filosporella annelidica*					
Quercus petraea		*Experiment food:* conditioned directly into the stream		35, 56 or 82 days	Before being cut in leaf discs of 1.2 cm in diameter and being frozen at −18°C	*Acclimation food:* ad libitum with plant detritus from the stream	Decomposition and feeding study	Danger et al. (2012)
Ulmus carpinifolia	Air-dried and leached for 96 h in distilled water and air-dried again	*Acclimation food:* decaying leaves *Experiment food:* incubated in soft water		*Experiment food:* 2–3 weeks	Before being given to the organisms	*Acclimation food*: ad libitum	Growth study	Hargeby and Petersen (1988)
Ulmus procera		*Experiment food*: different types. Depending on the experiment 1. Conditioned in stream water where leaf material from the river was added 2. Leaves soaked in tap water 3. Artificial pond water 4. Artificial pond water	*3. Anguillospora longissima* *Articulospora tetracladia* *Fusarium cavispermum* *Fusarium* spp. *Cylindrocarpon* spp. *Heliscus lugdunensis* *Lemonniera aquatica* *Tetracladium*	1. 14 days 2. 4 days 3. 15 days 4. 14 days	3. After being cut in leaf discs of 1.6 cm in diameter, but before drying at 60°C for 4 days 4. After being cut in leaf discs of 1.6 cm in diameter and after being dried at 60°C for 4 days		Feeding study	Graça et al. (1993a)

(continued)

Table 2 (continued)

Leaf species	Leaf storage	Conditioning water	Fungi species	Conditioning period	Conditioning time	Amount of leaves during acclimation	Type of study	Reference
			marchalianum *Tetracladium setigerum* *Triclodium angulatum* *4. Anguillospora longissimi*					
Ulmus procera		*Experiment food:* different types. Depending on the experiment 1. Conditioned in artificial pond water where leaf material from the river was added 2. Leaves soaked in tap water		1. 21 days 2. 4 days	After being cut in leaf discs of 1.6 cm in diameter and after being dried at 60°C for 4 days		Feeding study	Graça et al. (1993b)

organic material taken directly from the river as a natural source of fungi and bacteria (e.g. Zubrod et al. 2015, 2017) (see Table 2). Leaves can also be directly conditioned in situ by placing them in small nets/bags that are suspended in a river and retrieved after a specific number of days (Alonso et al. 2009; De Castro-Català et al. 2017; Forrow and Maltby 2000; Graça et al. 2001; Zubrod et al. 2015) (see Table 2).

Although river water might reproduce natural environmental conditions, it is sometimes contaminated, and this might have an impact. When river water is used, a chemical breakdown of the water should be undertaken and reported along with the study findings, so that any contamination is transparent. It is especially important to disclose if the river water is contaminated with the substance(s) under investigation in the study. If the test substances are present in the river water, the organism could be exposed to that concentration and also the experimental dose. Therefore, the organisms' response would not be a true reflection of the test concentration(s) but instead the reported dose combined with the concentration found in the river water. For example, contaminants might be absorbed onto the leaf surface and passed onto the organisms, or they could be released into the media, which might happen during the acclimation period and/or during the experiment itself, resulting in a compromised feeding activity. Therefore, the observed findings might be an indirect effect, due to the leaf quality and not as a direct result of the contaminant being tested.

The conditioning process usually takes around 2 weeks, but there are clear differences in the literature about this stage (see Table 2). The process ranges from a few days (Alonso et al. 2009), to several weeks (Blarer and Burkhardt-Holm 2016), and up to months (Danger et al. 2012) (see Table 2). When *Gammarus* spp. are offered a choice between leaves that have been conditioned for different periods of time, they prefer those that have been conditioned for the longest (Agatz and Brown 2014; Bird and Kaushik 1985). Consequently, experiments (in situ or ex situ) that provide the organisms with leaves that have been conditioned for a short or longer time period could potentially underestimate or overestimate the actual feeding activity of *Gammarus* spp.

It has been demonstrated that conditioned leaf material is more palatable (Agatz and Brown 2014; Graça et al. 1993b) and that different species of leaves (i.e. *Acer* spp. and *Ulmus* spp.), depending on the conditioning stage, might be more or less palatable compared to the others (Bird and Kaushik 1985). Consequently, it could be argued that it is impossible to compare experiments where organisms have been fed with organic material that has been conditioned for different periods of time. Organisms fed on leaves that have been conditioned for 1 week will probably eat less than those fed with the same leaves conditioned for 3 weeks, and leaf unpalatability might be mistakenly attributed to contaminant exposure. It has also been identified that *Gammarus pulex* fed with unconditioned leaves have a considerably lower respiration rate (Graça et al. 1993b).

Depending on the methodology used, the conditioning process might take place in different phases. There are studies where the leaves are provided to the organisms directly after the conditioning process (Bärlocher and Kendrick 1973a; Bloor 2010; Bundschuh et al. 2009; Forrow and Maltby 2000; Newton et al. 2018), whereas in

some cases the leaves are redried and soaked in water before feeding them to the organisms (Agatz et al. 2014; Blarer and Burkhardt-Holm 2016; Bundschuh et al. 2011b, 2013, 2017; Naylor et al. 1989), in order to prevent them from floating on the surface. In this case, the drying process requires the use of an oven, but unsurprisingly the time and temperature used vary between research groups. Bear in mind that the same food might be provided during the acclimation period and also during the experiment itself, unless the feeding experiment aims to study the feeding variation when a food source is either contaminated or compromised. In these studies, a specific contaminant or mixture of contaminants are usually incorporated during the conditioning process (Bundschuh et al. 2009; Hahn and Schulz 2007). When the conditioned leaves are oven-dried, they need to be resoaked in water before being provided to the organisms, in order to avoid floatation (e.g. Bundschuh et al. 2017; Zubrod et al. 2010). The water used to soften the leaves varies between research groups, and the water could act as a new source of contamination, especially if it differs from the one used during the original conditioning process.

4 Exposure and Feeding Rate Calculation

After the conditioning process and the acclimation period, the next step in a feeding study is the exposure itself. During this time, the Gammarids are exposed directly (i.e. the contaminant is in the water with the *Gammarus* spp.) (Zubrod et al. 2010) or indirectly (i.e. the contaminant is added during the conditioning process) (Bundschuh et al. 2009) to a contaminant, and their feeding behaviour is studied and estimated (see Table 3). These experiments might have different goals: they might be undertaken to either measure the changes in Gammarid feeding activity, Gammarid feeding preferences, or to study the effects on their growth. Consequently, the period of exposure could vary dramatically from a few hours (Bundschuh et al. 2011a) to a week (Felten et al. 2008a) or even several weeks (Weber et al. 2018), and sometimes fungal biomass analysis (estimated as ergosterol) and assimilation are incorporated, to strengthen the findings obtained from the feeding rate (Bundschuh et al. 2009; Newton et al. 2018).

Occasionally in feeding studies, the organisms undergo a period of starvation before the experiment is undertaken (De Castro-Català et al. 2017) (see Table 3). The main purpose of this starvation phase is to ensure that the organisms are at the same hunger state, but the duration of this phase varies in the literature. Once the experiment starts, the Gammarids are commonly provided with a precise amount of food, in other words the leaves provided have usually been dried, weighed and conditioned. This latter step, as previously mentioned, could have been carried out before the drying process or afterwards, so the final product could have different characteristics depending on the study. In order to provide the organisms with the same amount of food, the leaves are cut in small discs that range from a diameter of 0.7 to 4 cm depending on the research group (see Table 3). Before or after the conditioning process, the leaf discs are oven-dried for a specific period of time,

Table 3 Existing differences in the literature regarding feeding behavioural experiments

Leaf species	Contaminant	Water used for the experiment	Light/dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying temperature and time (before and after exposure)	Starvation	Equation used	References
Aesculus hippocastanum	Imidacloprid	Artificial pond water	12:12	1.6 cm 3 at the time and exchanged every 24 h	7 days (4 days exposure +3 days recovery phase)	*Leaves*: Before: 60°C for 48 h After: *Organisms*: After: 65°C for 48 h		$FR = \frac{F[t-1] - \frac{F[t]}{ld}}{G \times t}$ FR = feeding rate $F[t-1]$ = initial leaf dry weight (mg) $F[t]$ = final leaf dry weight (mg) G = dry weight of Gammarus (mg) ld = leaching decomposition factor t = time (days)	Agatz et al. (2014)
1. *Aesculus hippocastanum* 2. *Alnus glutinosa*		Artificial pond water	12:12	1. 1.6 cm 2. 2.0 cm	*Experiment 1*: 96 h *Experiment 2*: 9 days *Experiment 3*: 15 days	*Leaves*: 1. 2. Before: 60°C to constant weight After: *Organisms*: After: 90°C for 24 h Wet weight transform in dry weight using a linear regression		$FR = \frac{F[t-1] - \frac{F[t]}{ld}}{G \times t}$ FR = feeding rate $F[t-1]$ = initial leaf dry weight (mg) $F[t]$ = final leaf dry weight (mg) G = dry weight of Gammarus (mg) ld = leaching decomposition factor t = time (days)	Agatz and Brown (2014)
Alnus glutinosa	Microplastic debris	Mixed water (municipal water and softened water)		2.0 cm 2 discs at the time every 7 days	28 days	*Leaves*: Before: 60°C for 24 h After: 60°C for 24 h		$FR = \frac{L_i \times (CF) - L_f}{w \times t}$ FR = feeding rate L_i = initial leaf dry weight (mg) L_f = final leaf dry weight (mg) w = animal's average wet weight (mg) t = feeding time (days) CF = leaf change correction factor $CF = \frac{\left[\sum\left(\frac{C_f}{C_i}\right)\right]}{n}$ C_f= final dry weight of control leaf discs (mg) C_i= initial dry weight of control leaf discs (mg) n = number of replicates	Blarer and Burkhardt-Holm (2016)

(continued)

Table 3 (continued)

Leaf species	Contaminant	Water used for the experiment	Light/dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying temperature and time (before and after exposure)	Starvation	Equation used	References
Alnus glutinosa	In situ and ex situ feeding assays	In situ: river water Ex situ: river water		In situ: 5 leaf discs per cage of 1.6 cm in diameter Ex situ: 5 leaf discs per pot of 1.6 cm in diameter	6 days	*Leaves*: Before: After: 60°C for 4 days *Organisms*: After: 60°C for 4 days	24 h	$C = \frac{(L_1 \times C_L) - L_2}{W \times 6}$ C = feeding rate L_1 = initial leaves dry weight (mg) L_2 = final leaves dry weight (mg) W = organisms dry weight (mg) C_L = leaf weight change correction factor $C_L = \frac{\Sigma\left(\frac{C_2}{C_1}\right)}{N}$ C_1 = control leaf discs initial dry weight (mg) C_2 = control leaf discs final dry weight (mg) N = number of control replicates	Bloor and Banks (2006b)
Alnus glutinosa	Antibiotics (added during the conditioning process)	River water	Total darkness	1.5 cm 4 leaves at the time to assess food choice	58 h	*Leaves*: Before: 60°C for 24 h After: 60°C for 24 h *Organisms*: After: 60°C for 24 h	96 h	$C = \frac{\{[(f_b - f_a) - (n_b - n_a)] \times 24\}}{g \times t}$ C = leaf mass consumed f_b = initial dry mass of the leaf disc exposed to feeding (before conditioning) (mg) f_a = final dry mass of the leaf disc exposed to feeding (mg) n_b = initial dry mass of the leaf disc protected from feeding (mg) n_a = final dry mass of the leaf disc protected from feeding (mg) g = animal's dry weight (mg) t = feeding time (hours)	Bundschuh et al. (2009)
Alnus glutinosa	Fungicide tebuconazole			1.6 cm 4 leaf discs at the time, but only 2 discs were accessible to the organism	12 h	*Leaves*: Before: After: *Organisms*: After:			Bundschuh et al. (2011a)

Alnus glutinosa	Secondary treated wastewater	River water or secondary treated wastewater		2.1 cm 2 leaves at the time every 7 days	4 weeks	*Leaves*: Before: 60°C for 24 h After: 60°C for 24 h *Organisms*: After: 60°C for 24 h	$C = \frac{[L_b \times (k)] - L_a]}{g \times t}$ C = leaf mass consumed L_b = initial dry mass of the leaf disc (mg) L_a = final dry mass of the leaf disc (mg) g = animal's dry mass (mg) t = feeding time (days) k = leaf change correction factor $k = \frac{\sum\left[\frac{L_{ob} - L_{oc}}{L_{ob}}\right]}{n}$ L_{ob} = initial dry mass of control discs (mg) L_{oc} = final dry mass of control leaf discs (mg) n = number of replicates	Bundschuh et al. (2011b)
Alnus glutinosa	Antibiotics (added during the conditioning process and in the water with the Gammarids)	River and tap water mixture		1.1 cm 2 leaves at the time	24 days	*Leaves*: Before: 60°C for 24 h After: 60°C for 24 h *Organisms*: After: 60°C for 24 h	$C = \frac{[L_a \times (1-k) - L_b]}{g \times t}$ C = leaf mass consumed L_a = initial dry mass of the leaf disc (mg) L_b = final dry mass of the leaf disc (mg) g = animal's dry mass (mg) t = feeding time (days) $k = \frac{\sum\left[\frac{L_c - L_d}{L_c}\right]}{n}$ L_c = initial dry mass of control discs (mg) L_d = final dry mass of control leaf discs (mg) n = number of replicates	Bundschuh et al. (2017)
Alnus glutinosa	In situ feeding assay	River water		1.7 cm 4 leaf discs per cage	6 days	*Leaves*: Before: After: 60°C for 48 h *Organisms*: After: 60°C for 48 h	$C = \frac{(L_1 \times C_1) - L_2}{W \times T}$ C = feeding rate L_1 = initial leaves dry weight (mg) L_2 = final leaves dry weight (mg) W = organisms dry weight (mg) C_1 = leaf weight change correction factor T = feeding time (days)	Crane and Maltby (1991)

(continued)

Table 3 (continued)

Leaf species	Contaminant	Water used for the experiment	Light/dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying temperature and time (before and after exposure)	Starvation	Equation used	References
Alnus glutinosa	Ex situ and in situ feeding assay	Ex situ *experiment*: In situ *experiment*: River water	Ex situ *experiment*: 10:14 In situ *experiment*:	2.0 cm Ex situ*:* 20 discs per 20 gammarids In situ: 20 discs for 20 gammarids	Ex situ: 14 days In situ: 7 days	*Leaves*: *Before and after:* photo scanned		$FR_i = \frac{(S_{control} - S_i)}{\left(\frac{l_{i,0} + l_{i,t}}{2}\right) \times t}$ FR_i = feeding rate of replicate i $S_{control}$= total surface of the control leaf discs at the end S_i= total surface of the leaf disc at the end of replicate i t = feeding time (days) $l_{i,0}$ = number of living gammarids at the start $l_{i,t}$ = number of living gamamrids at the end	Coulaud et al. (2011)
Alnus glutinosa Quercus petraea		Filtered river water		1.0 cm 2 leaf discs at the time, with just one accessible to the organism	68–72 h	*Leaves*: Before: wet weight was used After: 65°C *Organisms*: After: 65°C			Danger et al. (2012)
Alnus glutinosa	Antidepressant and fungicide (added to the leaves during the conditioning or in the water)	Filtered river water	12:12	1.3 cm	14 days	*Leaves*: Difference between initial and final ash-free dry mass (AFDW)	24 h		De Castro-Català et al. (2017)
Alnus glutinosa	Metals: in situ experiment			2.1 cm 20 leaf discs at the time for each cage	14 days	*Leaves*: Before and after: photo scanned		$FR = \frac{\sum_{i=1}^{4} \frac{\left(S_{i,t0} - S_{i,t1}\right)}{\frac{l_{i,t0} + l_{i,t1}}{2}}}{7}$ FR = feeding rate $i = (i = 1 - 4)$ is the ith replicate S= total surface of leaf discs in each container (mm^2) l = number of living gammarids	Dedourge-Geffard et al. (2009)
Alnus glutinosa	Cadmium	Well water		2.0 cm	7 days	*Leaves*: Before: 105°C for 24 h After: 105°C for 24 h *Organisms*: After: 105°C for 24 h		$FR = \frac{\left(L_f \times C\right) - L_i}{WT}$ FR = feeding rate L_f = leaf final dry weight (mg) L_i = leaf initial dry weight (mg)	Felten et al. (2008a)

								W = organisms dry weight (mg) T = exposure time (days) C = leaching correction factor $C = \frac{\Sigma\left(\frac{L_b}{L_a}\right)}{8}$ L_b = final dry weight of control leaves (mg) L_a = initial dry weight of control leaves (mg)	
Alnus glutinosa		In situ: river water *Lab experiments*: artificial pond water			In situ: 6 days or 12 days *Lab experiments*: 6 days	*Leaves*: Before: (just wet weight) After: 60°C for 48 h *Organisms*: After: 60°C for 48 h		$C = \frac{(L_a \times C_L) - L_a}{W \times T}$ C = consumption of leaf material L_b = leaf discs initial dry weight (mg) L_a = leaf discs final dry weight (mg) W = organisms dry weight (mg) T = exposure time (days) C_L= correction factor of autogenic changes in leaf weight $C_L = \frac{\Sigma\left(\frac{Lc_a}{Lc_b}\right)}{N}$ Lc_a = control leaf discs final dry weight (mg) Lc_b=control leaf discs initial dry weight (mg) N = number of control replicates	Forrow and Maltby (2000)
Alnus glutinosa		Natural drilled groundwater		2.0 cm	21 days	*Leaves*: Photo scanned at the beginning and after 7 days when replaced		$FR = \frac{\Sigma_{i=1}^{N} \frac{(S_t - S_{t-1})}{\frac{n_t + n_{t-1}}{2}}}{T}$ FR = feeding rate T = total experiment duration (days) S_t=total surface of leaf discs in each container (mm^2) n_t = number of living gammarids	Geffard et al. (2010)
Alnus glutinosa	Antibiotics (added in the water during the conditioning process)	Pure natural water	Total darkness	2.0 cm	3 h	*Leaves*: Before and after: photo scanned *Organisms*: After: lyophilized for 18 h and stored in an exsiccator		Leaf area was calculated by counting the pixel numbers of each single leaf disc.	Hahn and Schulz (2007)
Alnus glutinosa		In situ: river water		Naylor et al. (1989)	6 days	*Leaves*: Before: (Naylor et al. 1989) After: 60°C for 4 days		$FR = \frac{(L_1 \times C_L) - L_2}{W \times 6}$ FR = feeding rate L_1 = initial leaves dry weight (mg) L_2 = final leaves dry weight (mg)	Maltby et al. (2002)

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Table 3 (continued)

Leaf species	Contaminant	Water used for the experiment	Light/dark cycle	Leaf disc size (diameter)	Length of the feeding study	Drying temperature and time (before and after exposure)	Starvation	Equation used	References
						Organisms: After: 60°C for 4 days		W = organisms dry weight (mg) C_L = leaf weight change correction factor	
Alnus glutinosa	Fungicides	SAM-5S	Darkness	1.6–2.0 cm	24 h 24 days	*Leaves*: Before: After: 60°C for 24 h *Organisms*: After: 60°C for 24 h		Calculated following the method of Zubrod et al. (2015)	Newton et al. (2018)
Alnus glutinosa	Anti-cholinesterase compounds			2.0 cm 5 for each tested concentration. Each beaker containing 20 organisms.		*Leaves*: Before: photo scanned and every 24 h		$FR_t = \frac{\sum_{i=1}^{5}\sum_{t=1}^{D}\left[\frac{S_{it}-S_{it-1}}{\frac{S_{it}+S_{it-1}}{2}}\right]/5}{D}$ FR_t = Feeding rate $i = (i = 1 - 5)$ is the ith replicate D = is the Dth day during the experiment period S = total surface of leaf discs in each beaker (mm^2) l = number of living gammarids	Xuereb et al. (2009)
Alnus glutinosa	Fungicides tebuconazole	River water		Prepared following the method of Bundschuh et al. (2011b) 2 leaf discs at the time	7 days	*Leaves*: Before: After: 60°C *Organisms*: After: 60°C		$C = \frac{L_b \times k - L_e}{g \times t}$ C = feeding rate L_b = initial dry mass of the leaf discs (mg) L_e = final dry mass of the leaf discs (mg) g = dry weight of Gammarus fossarum (mg) t = feeding time (days) k = leaf change correction factor $k = \frac{\Sigma((L_{ob} - L_{oe})/L_{ob})}{n}$ L_{ob} = initial dry mass of the leaf discs (mg) L_{oe} = final dry mass of the leaf discs (mg) n = number of replicates	Zubrod et al. (2010)

Alnus glutinosa	Fungicides	SAM-5S		3.0 cm 2 leaf discs at the time	7 days	*Leaves*: Before: 60°C for 24 h After: 60°C for 24 h *Organisms*: After: 60°C for 24 h		Calculate as described in Zubrod et al. (2010)	Zubrod et al. (2014)
Alnus glutinosa	Fungicides	Bioassay medium		2.0 cm	6 days	*Leaves*: Before: After: 60°C for 24 h *Organisms*: After: wet weight		$C = \frac{L_n - L_f}{t}$ C = consumption of leaf material L_n = dry weight of leaf discs prevented from feeding (mg) L_f = dry weight of leaf discs available for feeding (mg) t = exposure time (days)	Zubrod et al. (2015)
Populus sp.	Cadmium	Dutch standard water	Total darkness	Half a disc 3.0 cm in diameter	2 days	*Leaves*: Before conditioning: 60°C for 48 h After conditioning: 60°C for 48 h After experiment: 60°C for 48 h *Organisms*: After: 60°C for 48 h	4 days	$FA = \frac{F_i - F_f}{G}$ FA = feeding activity F_i = initial leaf dry weight (mg) F_f = final leaf dry weight (mg) G = dry weight of Gammarus (mg)	Alonso et al. (2009)
Populus tremuloides		River water	12:12	Whole leaves 48 leaves each	3 days	*Leaves*: Before: 45°C After: 45°C *Organisms*: After: 45°C		$RCR = \frac{\left[\frac{\text{mg food ingested}}{\text{d}}\right]}{\text{mg}}$ mean animal weight × 100 = %body weight weight consumed /day RCR = relative consumption rate	Arsuffi and Suberkropp (1989)
Quercus petraea	Microplastics	ISO medium		4 cm 1 disc every 8 days	48 days	*Leaves*: Before: 40°C After: 40°C *Organisms*: After: wet weight		Relative feeding rate calculate as mg leaf material consumed per mg body mass per day	Weber et al. (2018)
Ulmus procera		Artificial pond water		1.6 cm	1 day	*Leaves*: Before: 60°C for 4 days After: 60°C for 4 days *Organisms*: After: 60°C for 4 days		$FR = \frac{W_i - W_f}{t}$ FR = feeding rate W_i = mean weight of control discs (mg) W_t = final weight of leaf discs offered as food (mg) t = feeding period (days)	Graça et al. (1993a)

which is usually at the same temperature and for the same time period as used after the exposure (see Table 3). Once the leaf discs have been weighed, they are usually resoaked in water or conditioned, if that is still to be done, and provided to the Gammarids during the experiment, after sometimes rinsing with water.

During and after the exposure, data are collected to calculate the feeding rate of the organisms. The feeding rate equation is similar throughout the literature, but variations can still be found. For example, the data might not have been collected in the same way, even though the same equation might have been used. The most common way to estimate the feeding rate is to compare leaf dry weight before and after exposure to the amphipods, in relation to the duration of the experiment and the weight of the organisms. Commonly, the dry weight of the leaves is adjusted with a constant. This constant takes the loss in weight due to leaching and microbial decomposition into consideration. It is often calculated as the ratio of the control leaves final dry weight and their initial dry weight (e.g. Blarer and Burkhardt-Holm 2016) (see Table 3), but sometimes the equation might vary (e.g. Bundschuh et al. 2011b) (see Table 3). The control leaves are leaf discs that went through the same conditioning process, and through the same experimental conditions as those fed to the organisms, but they themselves were not.

The constant is not always positioned in the same place within the feeding rate equation. Most commonly, it multiplies with the initial dry weight of the leaves (e.g. Maltby et al. 2002) (see Table 3) as the initial dry weight might not be exact. A small amount of leaf might have been lost due to leaching and microbial decomposition during the conditioning process, for example. Sometimes it divides the final dry weight of the leaves (Agatz et al. 2014) (see Table 3). A proportion of the leaf might have been lost through leaching and the decomposition process, and not through Gammarids consumption. Both constant positions are trying to adjust the equation by compensating for the same problem, leaching and decomposition, but mathematically the equations are dissimilar and the results might be different.

Weight is sometimes considered as wet weight (Danger et al. 2012) or as ash-free dry weight (AFDW) (De Castro-Català et al. 2017) rather the normal dry weight (see Table 3). Once the exposure is complete, the leaf discs are collected and dried. The drying process is normally carried out in an oven and/or furnace (i.e. AFDW) at a specific temperature for a specific duration, which was also used for leaf disc preparation. As shown in Table 3, the temperature at which the leaves are dried can be very different and so can the duration of the process.

On rare occasions, the feeding rate is calculated by measuring differences in the leaf disc's surface area, which instead of being weighed are photographed and later analysed with a specific software (Coulaud et al. 2011; Hahn and Schulz 2007) (see Table 3). Scanning the leaf surface might result in very accurate data when it is calculated by pixel size or in mm^2, for example. This calculation does not incorporate a leaching constant (leaf change correction factor), which takes into account the loss of leaf weight due to the conditioning process. Differences in leaf surface could potentially occur as it happens with the loss in weight method. The authors acknowledge that it is still unclear if the choice of feeding equation and the different ways of calculating the feeding rate are actually comparable and equivalent. Interestingly

Coulaud et al. (2011) reported a relationship between the surface and the dry mass of their leaf discs, in order to facilitate possible comparisons between studies with different methodologies. Consequently, it is recommended that a leaf change correction factor should be calculated based on leaf surface loss, to take leaf conditioning changes into consideration and to make data from these different techniques more comparable.

5 Conclusions

Feeding behaviour has been used to investigate the sublethal effects of a wide range of contaminants over the years. As well as providing information on an organism level, feeding studies could also be adapted to understand the possible effects on entire populations, and therefore potential threats to a population could be transposed, to understand the prospective repercussions on the ecosystem.

Throughout this review, it is noticeable that there are variations within the adopted methodologies for the acclimation conditions, the leaf conditioning process and the leaf species used. This review has also highlighted that several different equations are used in the literature to quantify the feeding rate of Gammarids.

During the acclimation period, the organisms are kept at temperatures ranging from 10 to 22°C, even though all of the species considered in this review are from temperate countries. Temperature has been proven to have a significant impact on Gammarids by affecting their physiological parameters and their immune system. Temperature could ultimately have an impact on their feeding rate, which increases when the temperature is raised. The authors recommend that a constant temperature is maintained during the acclimation period and the experiment itself, in order to have a reliable estimation of the feeding rate, independent of a temperature difference. Moreover, the acclimation and experimental temperature should reflect the average conditions for the country where the experiment is being undertaken. In fact, both Maltby et al. (2002) and Coulaud et al. (2011) demonstrated that temperature has a major impact on feeding rate variability during in situ experiments. Consequently, when an in situ experiment includes several different deployments in different geographical areas, temperature should be measured in each location, so that the impact of temperature on the feeding rate can be estimated. Furthermore, the media in which the organisms are acclimated should always be aerated.

Similarly, the authors recommend that light/dark cycles aiming to reproduce seasonal conditions should be avoided, in order to allow the reproducibility of a study regardless of the time of the year. However, this is not the case for in situ studies. The temperature and light/dark cycles during acclimation for in situ experiments should best replicate the natural environment. Consequently natural light/dark conditions and the air and water temperature should be measured, reported and replicated.

Ex situ experiments should be standardised (e.g. using an artificial medium if possible), meaning that the medium's parameters (i.e. pH, conductivity, total

hardness) should be measured and reported, and the medium should be screened beforehand for contamination. If contamination is present, it is important to record the concentrations of the specific contaminant to identify the background level, to have a better understanding of the possible effects. This is especially noteworthy when river water is used to acclimate the organisms, in particular for in situ experiments where river water mimics natural environmental conditions for the acclimation period. It is difficult to say how long the acclimation should last. The authors recommend that further research is required to determine the impact of time frame on acclimation periods and to determine if a longer acclimation period results in stronger data with a lower level of variability.

Several different food types have been highlighted during this review, including different leaf species and conditioning methods. Even though Gammarids are biologically omnivorous organisms, a leaf-based diet is recommended in feeding studies, both during the acclimation period and the exposure, and the same food should be used for both (i.e. same leaf species and same preparation). *Alnus* spp. are the most commonly used leaf material for freshwater Gammarid feeding studies. The authors therefore recommend *Alnus* spp. as a standardised food source for ecotoxicological assays. However, the distribution of *Alnus* spp. is not ubiquitous around the world, and therefore it might be challenging for some researchers to source them for their experiments. In such situations, industrial feed might be a better solution to overcome the problem of non-standardisation. If leaf material is used, applying a conditioning process is recommended, since conditioned material has been proven to be more palatable and have a lower C:N, which translates to a better energy supply. Moreover, it has also been demonstrated how leaf palatability increases when they are conditioned for longer time periods. Consequently, a short conditioning period (i.e. a few days) should be avoided, and organisms should be fed on leaves conditioned for at least 10 days. However, this time period should be prolonged if using fresh leaf material, since it has been reported that conditioning takes longer. In ex situ experiments, conditioning should be conducted using an artificial media inoculated with *Cladosporium* spp., which is the most common fungi species used in the literature to condition leaves. This will ultimately reduce the likelihood of contamination that might result from using river detritus as a source of fungi inoculum for conditioning leaves.

On the other hand, for in situ studies, the conditioning process should ultimately replicate, as accurately as possible, real-life environmental conditions and processes, which means using river water, inoculum and Gammarids from the study site. As previously mentioned, the composition of the water needs to be identified and also the chemical parameters; the latter could then be replicated during the conditioning process. For in situ experiments, the authors recommend conditioning the leaf material directly in the river. For example, placing leaves in small net bags that are submerged and secured in the river where the experiment would take place. This would provide the Gammarids with the same type of food during the acclimation period and exposure. However, conditioning takes time, so it should be undertaken well in advance of the experiment.

It is still unclear if conditioning should take place before or after the leaves are cut into discs, dried in the oven and weighed. Consequently, the authors recommend that further investigations need to be undertaken to compare if drying the leaf discs in the oven should be undertaken before or after the conditioning process and if either of these methodologies alter the feeding rate of Gammarids. Organisms are usually fed ad libitum during the acclimation period. To further reduce the inner variability and strengthen the data, the authors recommend incorporating a starvation period in the experimental design. This starvation period should take place before the feeding experiment, and its purpose is to synchronise the organisms' hunger levels. The authors also recommend that organisms of a comparable size range should be used in experiments as it has been proven that Gammarids of different sizes have a different feeding rate. Juveniles are more sensitive to contaminants, but their feeding rate is characterised by a higher variability over time, which makes them more suitable for short-term studies. On the other hand, because of their greater sensitivity, juveniles are better for ecotoxicological studies by providing ecologically relevant risk assessments for contaminants. *Gammarus* spp. has been widely adopted for ecotoxicological studies, but the genus contains many different species, and even though very similar, there are still differences in their sensitivities, meaning that the choice of one species over the other should be carefully considered, depending on the contaminant tested.

This is of particular interest for in situ experiments since the adopted species would be dependent on the site, but also dependent on the season, which could determine the availability of particular organism sizes. So in order to further reduce inner variability and allow better estimation of the feeding rate, organisms should be measured at the start of an experiment, possibly by photography and length measurements, in order to have a pool of organisms of the same size and potentially the same life stage. This is particularly noteworthy when growth is measured alongside the organisms feeding rate.

The source of the organisms might also have an impact on the results. Organisms collected in the wild could be better suited for in situ studies, as they could provide a more realistic site-specific response. However, local site-related species may be characterised by previous exposure histories that could ultimately influence their feeding rate (e.g. they could potentially be acclimated to a certain level of pollution). This is a problem that has to be taken into consideration both for in situ and ex situ experiments. Perhaps laboratory-bred organisms should be used to reduce variability even further, and it would provide a constant stock of Gammarids (Blockwell et al. 1996; Bloor and Banks 2006a, b; McCahon and Pascoe 1988). However, breeding Gammarids is not always possible and it is highly species dependent. Long-term culturing could also potentially lead to a higher or lower contaminant sensitivity and a reduced genetic variability.

The last step of a feeding study involves the quantification of the feeding rate by using an equation. As highlighted in this review (see Table 3), there are various equations in the literature that are indiscriminately used to calculate the feeding rate. However, some of these equations are mathematically different, and it raises the question, are the equations and the feeding rates generated by them equal?

The feeding rate can be estimated by using the leaf weight or surface area. The equation that is most commonly adopted estimates the feeding rate by comparing leaf dry weight before and after being provided to the Gammarids, divided by the time (expressed in days) and the weight of the organisms. Usually the dry weight of the leaf discs is adjusted with a constant. The authors recommend that the position of the constant is dependent on when the leaves are conditioned and dried. If the leaves are conditioned before being dried and weighed, the constant should multiply with the initial dry weight, so that it takes into consideration that the leaf disc might have lost more weight through being submerged in water during the experiment. However, if the leaf discs are dried and weighed before being conditioned, the final dry weight should be divided by the constant, because some of the leaf material might have been lost through leaching and not through Gammarid feeding.

Another consideration is that the constant is not always calculated in the same way, and this could ultimately alter the experimental results. Again, the authors recommend that further research is required to understand the impact of the various constant positions on the outcome of a study. Until then, the authors recommend that the equation provided by Maltby et al. (2002) is adopted, as it is representative of real-life environmental feeding.

When leaf area is used to calculate the feeding rate, the constant is not often included in the equation. This means that the possible loss of leaf material due to the leaching process is not taken into consideration. Leaf area is often used to calculate the feeding rate for in situ experiments, so the authors recommend that if this method is going to be used, a set of control leaves should also be established, in order to calculate a leaching constant based on the difference in surface area.

It is clear that a standardised protocol is required, which would benefit the scientific community and regulatory authorities and allow them to interpret and compare published literature to understand the impact of various contaminants (and mixtures) on the environment. This could be achieved by undertaking serial experiments to clarify what impact these heterogeneities have on the final results. There are methodologies such as Naylor et al. (1989) and Nilsson (1974) that have been used many times, but unfortunately, there are still others that are the result of a mixed methodology. The variability within feeding studies has already been acknowledged, and the first steps towards standardisation have evolved (Agatz and Brown 2014).

A standardised ex situ methodology would greatly benefit this field of research, by not only allowing a more meaningful comparison between the peer-reviewed literature, but also to better understand the impact that specific contaminants could have on Gammarid populations and ecosystems. This could be enhanced further if ex situ experiments are placed side by side with biomarker analysis and in situ studies. In theory, in situ tests could provide a realistic and integrated understanding of real environmental pollution. If standardised, in situ tests could be used by regulators to critically evaluate the state of an ecosystem and the potential impact that a certain contaminant or mixture could have on the environment. This is of particular interest since the establishment of the Water Framework Directive (European Union 2000), which outlines that all European water bodies should reach ‘good quality status’ by

2015 and has since been extended. The establishment of a standardised suite of in situ and ex situ feeding assays would provide a realistic monitoring tool and environmental risk assessment, which would be of benefit to the scientific community, and also decision makers.

6 Summary

Feeding behaviour of freshwater Gammarids has been used for several decades as a sublethal toxicity endpoint. Feeding behaviour has been demonstrated to be an effective endpoint, but there is not a standardised assay. This paper aims to review the existing published literature to highlight the methodological discrepancies in feeding behavioural studies (both in situ and ex situ). Key discrepancies in the acclimation period are temperature, duration, media, light/dark cycles and the characteristics of the test organisms. Interestingly, the food preparation method and the choice of feeding rate equation are also diverse. Non-standardisation of any of these factors could influence the outcome of the experiment and render a comparison between studies difficult. There is an undeniable need for scientific discussion and agreement on a standardised protocol for feeding behavioural studies, to ensure that all future studies are directly comparable and to enhance the usefulness of feeding assays as a biomonitoring tool to assess water quality.

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Appendix B

Appendix B1: Abstract for Poster presentation, SETAC 28th Annual meeting in Rome, May 13th-17th , 2018.

Impact of antibiotics on the feeding rate of the freshwater shrimp *Gammarus pulex*

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Antibiotics are one of the main class of pharmaceuticals and their consumption is grown exponentially from their first discovery in 1920s and consequently also their release in the environment. Subsequently natural bacterial and fungal communities can be affected along with organisms that rely on them as a food source, such as the freshwater detritivore *Gammarus pulex*, that commonly feeds from naturally conditioned leaf material

This project aims to evaluate the alteration of the feeding rate of the sensitive freshwater species *Gammarus pulex* after being fed with antibiotic-conditioned *Alnus glutinosa* leaves. To do this, four different antibiotics were tested singularly, Ciprofloxacin (CIP) and Tetracycline (TET), or as a mixture, Sulfamethoxazole (SMZ) and Trimethoprim (TMP).

First, air dried *Alnus glutinosa* leaves were conditioned with river water and environmentally realist concentration of antibiotic for 14 days (200 μg/L, 20 μg/L and 2 μg/L). River water was changed after 7 days and the concentration of the pharmaceutical compound or mixture re-established afterwards.

Specimens of *Gammarus pulex* were kept for 12 days at 15˚C under a 12:12-h light:dark cycle. After a 48 hours starvation period in the dark they were fed with antibiotic conditioned leaf discs for 24 hours under a 12:12-h light:dark cycle.

To evaluate the possible alteration to the feeding rate (FR) of the freshwater shrimps *Gammarus pulex*, changes in shrimps' dry weight and leaf consumption were measured to assess the leaf mass consumed. Photos of each leaf disc was also taken and subsequently analysed to measure the leaf area consumed.

Results show that the feeding rate of *Gammarus pulex* is not altered when exposed to TET at any of the concentration tested, whereas tend to statistically increase (*Kruskal-wallis test*; X^2=13.239; p=0.004. *Friedman test;* X^2=11.960; p=0.008) when the leaf discs are conditioned at the lower (2 μg/L) and median (20 μg/L) concentration of the antibiotic mixture of SMZ and TMP compared to the control and the higher concentration (200 μg/L). This result were partially backed up by the photo analyses. Difference in leaf area consumed was not overall significant (*Kruskal-wallis test*; X^2= 3.449; p=0.327. *Friedman test*; X^2=4.360; p=0.225), but it appeared to be significant once the individual concentrations were singularly compared. The area consumed of the leaf discs exposed to the lower concentration (2 μg/L) is significantly higher than the discs conditioned with the higher mixture concentration (200 μg/L), (*Wilcoxon Signed Ranks test*; p=0.027).

The non-stop release of antibiotics into the aquatic ecosystems is not only a prospective hazard for public health, but it may be considered nowadays an actual risk for the environment, because the massive consumption of antibiotics is leading to an alarming proliferation of antibiotic resistance pathogens, which is not only a threat for the human health but also to all those species that rely on microbial communities as a food source.

<u>Appendix B2</u>: Certificate for the attendance and presentation of a poster at the SETAC 28th Annual meeting in Rome, May 13th-17th , 2018

We certify that

Giulia Consolandi

(University of Portsmouth, United Kingdom)

attended the
SETAC Europe 28th Annual Meeting in
Rome, Italy on 13-17 May 2018

And presented a **Poster presentation**

Entitled:
"Impact of antibiotics on the feeding rate of the freshwater shrimp Gammarus pulex"

In Session: Antibiotics and Antibiotic Resistance in the Environment: Fate and Ecological Effects, Resistance Development and Implications for Human Health (P)

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Appendix B3: Abstract for Platform presentation, SETAC 28th Annual meeting in Rome, May 13th-17th , 2018.

Impact of antibiotics on the feeding rate of the freshwater shrimp *Gammarus pulex*

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1. Introduction

Antibiotics are one of the main categories of pharmaceuticals and their release into the freshwater environment can impact the natural bacterial and fungal communities, which can threaten the survival of organisms that rely on them as a food source. One such organism is the freshwater detritivore *Gammarus pulex* that commonly feeds on detritus such as, naturally conditioned *Alnus glutinosa* leaves [1]. The study aim was to estabilsh if the feeding rate of *Gammarus pulex* was altered when their food source (*Alnus glutinosa*) was exposed to environmentally realistic concentrations of antibiotics during the natural leaf conditioning process. The investigation included three antibiotic scenarios (1) exposure to the bacteriostatic agent Tetracycline (TET), (2) exposure to a mixture of Sulfomethoxazole (SMZ) and Trimethoprim (TMP) bacteriostatic agents that are commonly prescribed together and (3) exposure to the bactericidal agent and broad-spectrum antibiotic Ciprofloxacin (CIP).

2. Materials and methods

Stream water and adult specimens of *Gammarus pulex* (n=60) were collected from an unpolluted stream in Emsworth, Hampshire, (50°51'38.7''N, 0°55'43.9''W) and transported to the Institute of Marine Science, University of Portsmouth. For acclimatisation purposes, the organisms were kept at 15°C under a 12:12 h light:dark cycle in a 3 L plastic aquarium filled with charcoal filtered tap water for 10 days prior to the start of the investigation. During this period the organisms feed on a plentiful supply of naturally conditioned *Alnus glutinosa* leaves [2]. Naturally abscised *Alnus glutinosa* leaves were handpicked from Sir Harold Hillier Gardens, Romsey, Hampshire (51°00'45.7''N, 1°27'55.2''W), air dried and stored at 18°C until required.

The organisms were subsequently starved for 48h in the dark before each feeding experiment. Air dried *Alnus glutinosa* leaves were cut into 60 discs of 1.3 cm Ø, dried at 60°C for 24 h and weighed [1,3]. For 7 days the discs were naturally conditioned by soaking them in 5 ml of stream water that was inoculated with a 200 μg/L, 20 μg/L and 2 μg/L antibiotic treatment, and a stream water control was also established (15 discs per treatment). After the initial 7 day period, the stream water and antibiotic inoculum were renewed and left for a further 7 days. Once conditioned, each disc was thoroughly rinsed with charcoal filtered water, photographed and put into a small plastic pot containing 100 ml of charcoal filtered water and a *Gammarus pulex* for 24 h under a 12:12 h light:dark cycle. After which time, the leaves were re-photographed and the *Gammarus pulex* sacrificed by exposure to -20°C temperature. The non-consumed leaf material and sacrified *Gammarus pulex* were then dried at 60°C for 24 h and weighed. The leaf disc before and after photographs were subsequently analysed with Image J software in order to calculate the area consumed. This protocol was performed with antibiotic scenario 1, 2 and 3.

3. Results and discussion

The data was handled using IBM SPSS software version 24. In the first instance, the normailty of the data was tested and in some instances normality was confirmed (p=>0.05) while in others cases the data was not normal. Consequently, a battery of paramentric and non-parametric tests were applied as deemed appropiate (p=<0.05). Scenario 1, exposure to Tetracycline (TET) showed there was no significant difference in the amount of leaf consumed between the concentrations (ANOVA: Z=0.198, p=0.897) or the leaf area consumed (ANOVA: Z=0.473, p=0.702), (Figure 1 A & B) that indicates Tetracycline (TET) was not a concern in relation to feeding at environmentally realistic concentrations. Scenario 2, exposure to a mixture of Sulfomethoxazole (SMZ) and Trimethoprim (TMP) showed a significant difference in leaf consumed (Kruskal-Wallis: Z=13.239, p=0.004) between all the concentrations except the control & 200 μg/L (Wilcoxon: Z=-0.682, p=0.496) and 2 μg/L & 20 μg/L (Wilcoxon: Z=-0.909, p=0.363), (Figure 1 C). However, there was

no significant difference in the leaf area consumed (Kruskal-Wallis: Z=3.449, p=0.327) except for 2 μg/L & 200 μg/L concentrations (Wilcoxon: Z=-2.215, p=0.027), (Figure 1 D). The results of this mixture investigation demonstrate that there was an impact on the *Gammarus pulex* feeding rate but further investigation would be required to identify the full extent. Scenario 3, exposure to Ciprofloxacin (CIP) showed there was no significant difference in the amount of leaf consumed between the concentrations (Kruskal-Wallis: Z=1.568, p=0.667) or in the leaf area consumed (Kruskal-Wallis: Z=7.572, p=0.056) expect for the control & 2 μg/L (Wilcoxon: Z=-2.726, p=0.006) and the control & 200 μg/L (Wilcoxon: Z=-2.613, p=0.009), (Figure 1 E & F), which suggested Ciprofloxacin (CIP) was also not a concern at environmentally realtistic concentrations.

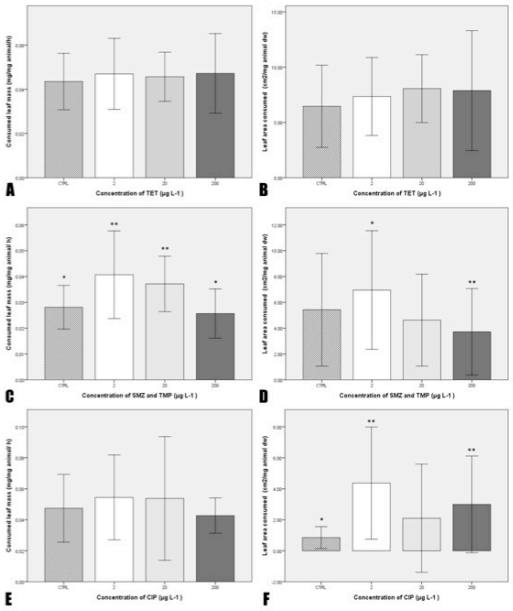

Figure 1 A, C & E showed the amount of leaf material consumed by *Gammarus pulex* when exposed to a 200 μg/L, 20 μg/L and 2 μg/L or a filtered water control. **Figure 1 B, D & F** showed the leaf area consumed by *Gammarus pulex* when exposed to a 200 μg/L, 20 μg/L and 2 μg/L or a filtered water control. **(A & B)** exposure to Tetracycline (TET), **(C & D)** exposure to a mixture of Sulfomethoxazole (SMZ) and Trimethoprim (TMP) and **(E & F)** exposure to Ciprofloxacin (CIP). The mean values are presented and the bars represent the standard deviation.

4. Conclusions

In conclusion, from a feeding prospective, environmentally realistic concentrations of Tetracycline (TET) and Ciprofloxacin (CIP) are not a concern. Whereas, exposure to a mixture of Sulfomethoxazole (SMZ) and Trimethoprim (TMP) did influence the feeding rate of *Gammarus pulex*, which might be due to their combined action. However, further research would be required to investigate these drugs individually to identify if these result were driven by one or the combination, and also to establish if there is a genuine environmental concern associated to this mixture or if the data is blurred in some way.

5. References

[1] Bundschuh M, Hahn T, Gessner MO., Schulz R. 2009. Antibiotics as a chemical stressor affecting an aquatic decompose-detritivore system. Environ. Toxicol. Chem. 28: 197-203.

[2] Bloor M. 2010. Animal standardisation for mixed species ecotoxicological studies: Establishing a laboratory breeding programme for *Gammarus pulex* and *Asellus aquaticus*. Zoologica Baetica. 21, p. 179-190.

[3] Maltby L, Clayton SA., Wood RM., McLoughlin N. 2002. Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: robustness, responsiveness, and relevance. Environ. Toxicol. Chem. 21: 361-358.

<u>Appendix B4</u>: Certificate for the attendance and platform presentation at the SETAC 28th Annual meeting in Rome, May 13th-17th , 2018

ROME 2018

We certify that

Giulia Consolandi

(University of Portsmouth, United Kingdom)

attended the
SETAC Europe 28th Annual Meeting in
Rome, Italy on 13-17 May 2018

And presented a **Platform presentation**

Entitled:
"Impact of antibiotics on the feeding rate of the freshwater shrimp Gammarus pulex"

In Session: Antibiotics and Antibiotic Resistance in the Environment: Fate and Ecological Effects, Resistance Development and Implications for Human Health

authors:
Giulia Consolandi, Michelle Bloor, Alex Ford

Bart Bosveld
SETAC Europe Executive Director

SETAC EUROPE
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Appendix B5: Abstracts for Poster presentation, SETAC 29th Annual meeting in Helsinki, May 26th-30th , 2019.

Using the Multispecies Freshwater Biomonitor to assess the potential impact of the antidepressant Venlafaxine on the amphipod *Gammarus pulex*

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[2]Institute of Marine Sciences, School of Biological Sciences, University of Portsmouth, Ferry Road, Portsmouth, PO4 9LY, UK

[3]LimCo International GmbH, Wollmatinger str. 22, Konstanz, D-78467
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The Serotonin and Norepinephrine Reuptake Inhibitor (SNRI) Venlafaxine (VEN) is an antidepressant that is often prescribed to treat depression and anxiety. VEN has been frequently detected in aquatic environments where its possible effects are still widely unknown. The aim of this study was to investigate the potential impact of VEN at environmentally realistic concentrations (20 ngl^{-1}, 2 μgl^{-1} and 20 μgl^{-1}) and identify if it effects the behaviour of the freshwater amphipod *Gammarus pulex*. River water and specimens of *Gammarus pulex* were collected from a non-polluted stream in Allensbach, Germany (47°42'27.6'' N, 9°06'26.5''E) during September 2017. The organisms were acclimated for 14 days at 18°C in aerated stream water in the dark. *Gammarus pulex* were provided with *Alnus glutinosa* leaves. In order to test the effects of the antidepressant VEN a serial exposure was undertaken. Specimens of *Gammarus pulex* (n=30) were used for each concentration (20 ngl^{-1}, 2 μgl^{-1} and 20 μgl^{-1}) and a control. 5 x 250 ml glass beakers were filled with 100 ml of each VEN concentration. 6 *Gammarus pulex* were put into each beaker with a 3 cm Ø *Alnus glutinosa* leaf disc. Behavioural analyses were carried out using the Multispecies Freshwater Biomonitor (MFB).The MFB is a device that tracks and records the behavioural activity of different aquatic organisms through a quadruple impedance conversion technique. Each organism was placed in a small plastic chamber in a 4 L

aquarium. The presence of electrodes inside the chamber enables an organism's movement to be recorded as a change in the electrical field. A movement generates specific frequencies which in the case of *Gammarus pulex* are from 0.5 to 2.5 Hz. *Gammarus pulex* swimming behaviour was recorded 5 times over a period of 12 days. The data was analysed with a general linear effects model and it was found that there was a significant difference in their behaviour over time (F=4.107; p=0.004) and that there was a significant different in their behaviour between treatments (F=4.364; p=0.006), but there was no interaction between treatments and time (F=1.092; p=0.372). Pairwise comparisons showed a significant difference between the lowest concentration (20 ngl^{-1}) and the control (p=0.011) and the median concentration (2 μgl^{-1}),(p=0.012). Overall, VEN does have an impact on the behaviour of *Gammarus pulex,* but this effects is not related to time.

Effects of the antidepressant Venlafaxine on the feeding rate and behaviour of the freshwater amphipod *Gammarus pulex*

Giulia Consolandi[1], Alex Ford[2] and Michelle Bloor[1]

[1]School of Earth and Environmental Sciences, University of Portsmouth, Burnaby building, Burnaby Road, Portsmouth, PO1 3QL, UK

[2]Institute of Marine Sciences, School of Biological Sciences, University of Portsmouth, Ferry Road, Portsmouth, PO4 9LY, UK
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In the last decade the prescribing of antidepressants has increased dramatically. Venlafaxine (VEN) is one of the most commonly detected antidepressants in European streams. Little is known about the potential impact of VEN on non-target aquatic organisms at enviromentally realistic concentrations but it is hypothesised to interfere with behaviour and feeding. We aim to identify if the behaviour and/or feeding rate of *Gammarus pulex* a sensitive freshwater macroinvertebrate was altered when exposed to environmentally realistic concentrations of VEN for 7 days. Specimens of *Gammarus pulex* were kept for 14 days at 15˚C under a 12:12 h light:dark cycle. After a 48 h starvation period

they were exposed for 7 days to 3 different concentrations of VEN (20 ngl^{-1}, 2 μgl^{-1} and 20 μgl^{-1}). To evaluate the possible alteration on the feeding rate (FR), *Alnus glutinosa* leaves were cut into discs, dried and weighted. The leaf discs were then conditioned in stream water for 14 days, after which time each disc was photographed and given to the organisms. Each organism was provided with 2 discs at a time, that were replaced every 2 - 3 days. The leaves were then re-photographed, dried and weighted again. The before and after photographs were analysed to determine the leaf area consumed, whereas changes in the leaf disc weight were used to calculate the consumed leaf mass. To quantify possible changes in the swimming velocity, *Gammarus* pulex behaviour was recorded after 24 h and 7 days by using a DanioVision™ observation chamber. Data were analysed with a linear mixed effects model (p=<0.05). *Gammarus pulex* FR was not altered. There was no significant difference between the concentrations in either the consumed leaf mass (F=0.764, p=0.519) or the leaf area consumed (F=2.610, p=0.060). The interaction between treatments and time was not significant (F=0.726, p=0.630; F=1.884, p=0.090). However there was a significant overall change in FR over the length of the experiment, but unrelated to the concentration tested (F=14.742, p=0.000; F=62.221, p=0.000). Alterations in velocity were not significant between the concentrations (F=0.934, p=0.430). A significant interaction was measured between concentration and exposure time (F=26.025, p=0.000), meaning that the organisms velocity was different across the concentrations after 24 h and 7 days. Overall this study showed that VEN may affect *Gammarus pulex* behaviour when exposed to environmentally realistic concentrations for 7 days.

Appendix B6: Certificate for the attendance and presentation of a poster at the SETAC 29th Annual meeting in Helsinki, May 26th-30th , 2019

We certify that

Giulia Consolandi

(Uni of Portsmouth, United Kingdom)

attended the

SETAC Europe 29th Annual Meeting
from 26 –30 May 2019 in Helsinki, Finland

Bart Bosveld
SETAC Europe Executive Director

Appendix C

Appendix C1:

Methodology to quantify leaf surface area by using ImageJ Software

For each feeding experiment that was carried out during the current PhD project, two different methodologies were used to quantify the amount of leaf consumed by each specimen of *G. pulex*. One of these methodologies involved taking photos of each leaf disc with a stereomicroscope Leica S8 APO B (Figure A.1) and calculate the surface area by using the pixel size of the picture.

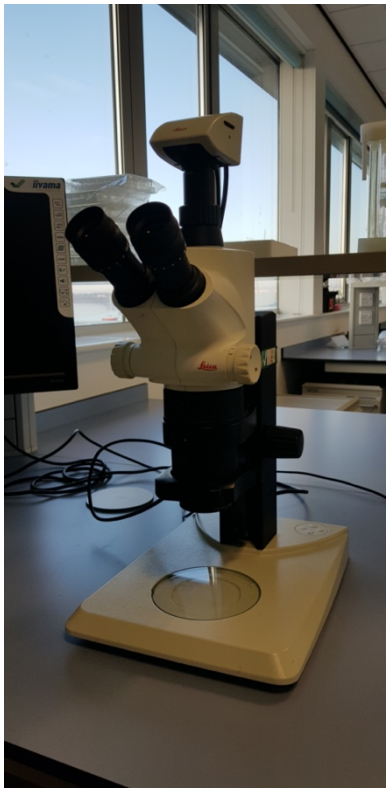


Figure A.1. Stereomicroscope Leica S8 APO B

In order to calculate the pixel size of the picture the following equation was used:

$$Picture\ pixel\ size = \frac{camera\ pixel\ size * binning}{(objective\ magnification) * (lens\ magnification) * (C\ mount)}$$

Where:

$camera\ pixel\ size = 3.34\ \mu m$

$binning = 1x1$

$objective\ magnification = 1x$

$lens\ magnification = 0.63$

$C\ mount = 0.5$

Consequently:

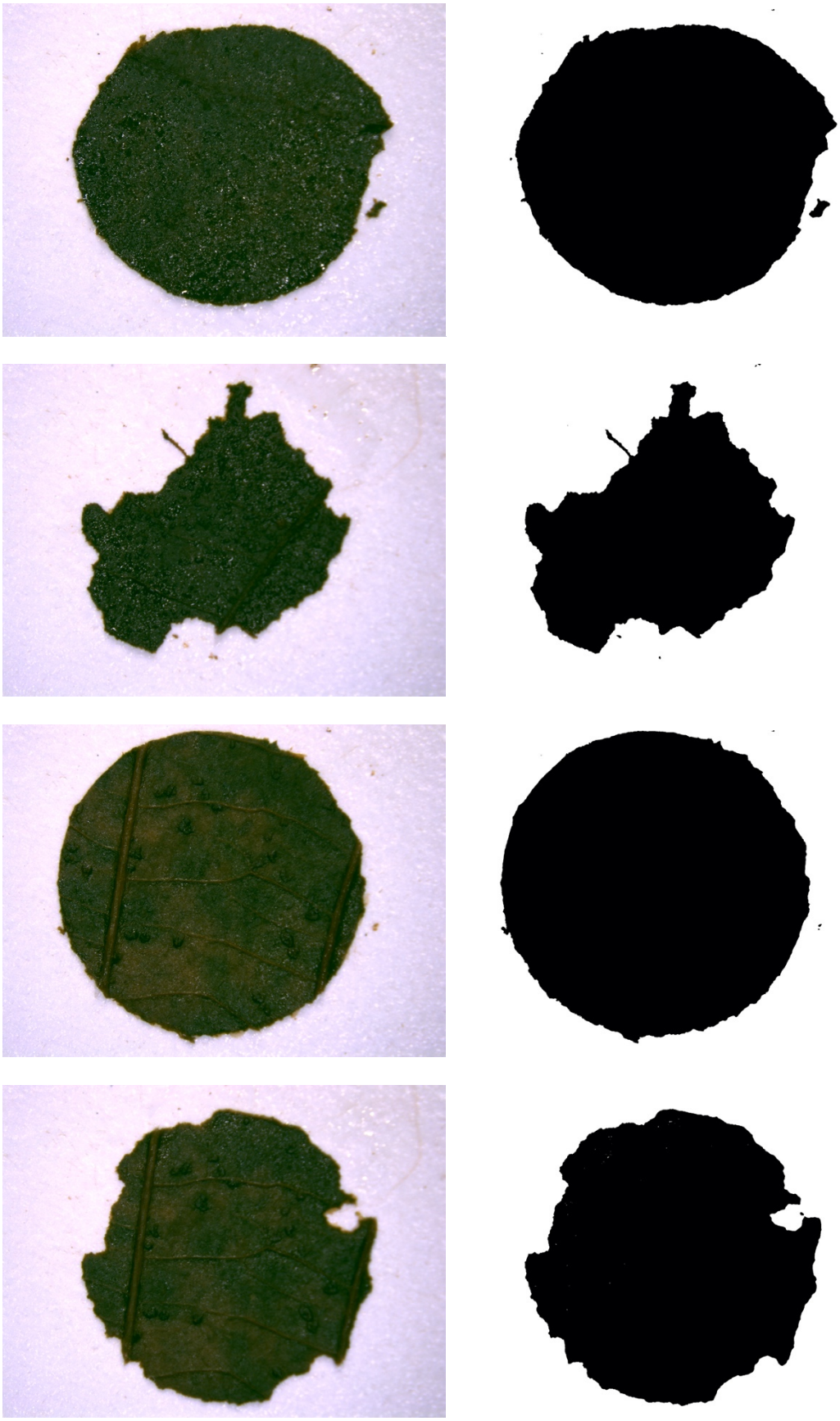
$$Picture\ pixel\ size = \frac{3.34\mu m\ *(1x1)}{(1x)*(0.63)*(0.5)} = 10.60\ \mu m$$

The $Picture\ pixel\ size$ was then used to calibrate the image and to calculate the surface area in μm^2.

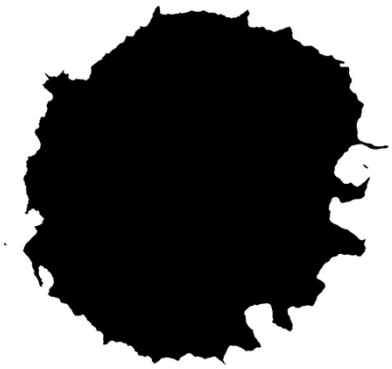
In order to quantify the leaf surface area the image was first transformed in a 8 bit image and then in a binary photo. Finally, by using a selecting tool, the area was selected and calculated by the software. Examples of before and after photos can be found in Appendix C2.

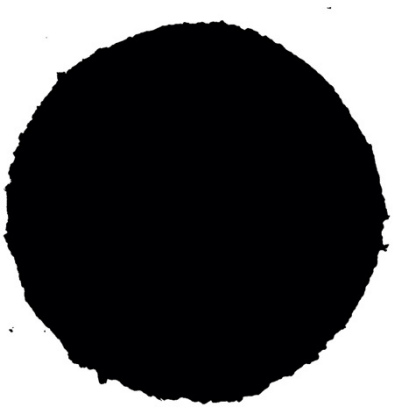
Appendix C2:

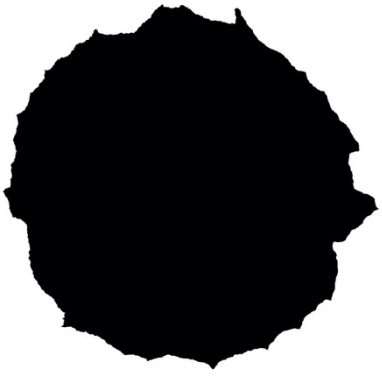
Examples of before and after photos of *Alnus glutinosa* leaf discs:

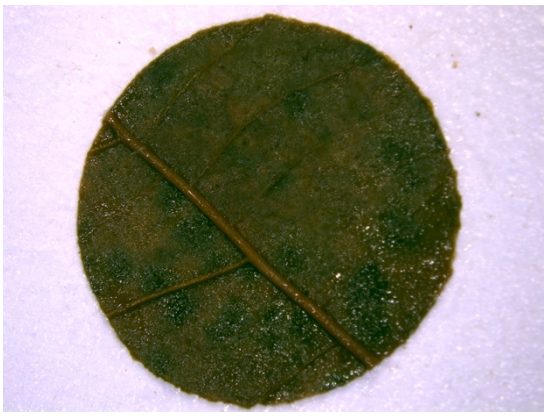


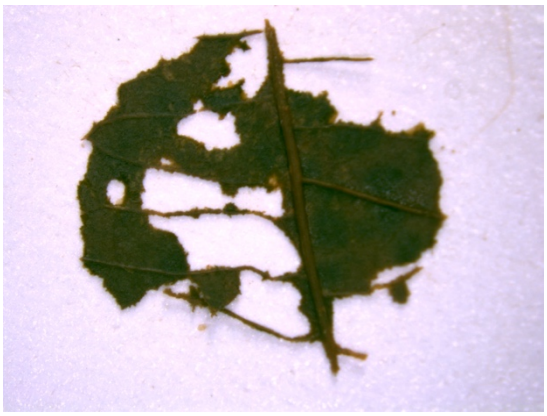


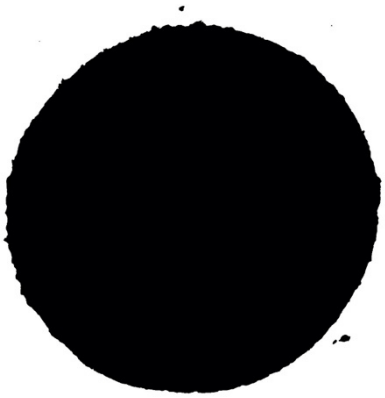
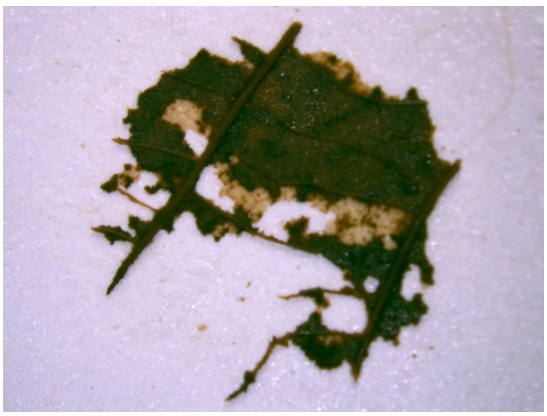












Appendix D

Prüfbericht

Technologiezentrum Wasser | DVGW

DVGW-Technologiezentrum Wasser, Karlsruher Str. 84, 76139 Karlsruhe

Auftraggeber **University of Portsmouth**
Burnaby Road
Portsmouth

Probenehmer Auftraggeber

Parameter Venlafaxin
Verfahren PV M 3000/0
Einheit µg/L

Probenahme	Probeneingang	Probenbezeichnung	BG	Ergebnis	Grenzwert
Probe-Nr.					
18.10.2017	19.10.2017	20 ng/L	0,010	2,5	
2017017232					
18.10.2017	19.10.2017	2 µg/L	0,20	1,5	
2017017233					
18.10.2017	19.10.2017	20 µg/L	2,0	16	
2017017234					
18.10.2017	19.10.2017	20 mg/L	2000	16000	
2017017235					

Bemerkung:

BG = Bestimmungsgrenze

Die Ergebnisse beziehen sich ausschließlich auf die untersuchte Probe.

Untersuchungsende, Karlsruhe, den 26.10.2017

A. Thoma
Dipl.-Geoökol. A. Thoma
Gruppenleiterin

Prüfbericht Nr. 000186201

Seite 1 von 1

Appendix E

19 October 2018

University of Portsmouth

Professor Matt Guille
School of Biological Sciences
King Henry Building
King Henry I Street
Portsmouth PO1 2DY
England

Tel: +44 (0)23 9284 2047
Fax: +44 (0)23 9284 2070
email: matthew.guile@port.ac.uk

Dear Miss Consolandi,

RE: Ethics submission – S**hrimp collection, maintenance in the laboratory and use for research experiments.**

Approval of project by the Animal Welfare and Ethical Review Body (AWERB)

I am very happy to confirm that we were able to fast track your application and that the AWERB gave its approval for your proposal concerning work within the above project.

The AWERB uses UK Home Office guidelines on the Animals (Scientific Procedures) Act 1986 when assessing proposals and adheres to the regulations of the European Directive 2010/63/EU. Your project has been assessed as not falling within A(SP)A because it uses invertebrates. We are confident that the proposal demonstrates appropriate consideration of the Three Rs and animal welfare. Please use this letter as confirmation of ethical approval from AWERB, University of Portsmouth. Please use the number 1018A as confirmation of the successful review.

Yours sincerely,

MJ Guille PhD FSB
Professor of Developmental Genetics and Chair, AWERB

FORM UPR16

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information

Postgraduate Research Student (PGRS) Information			**Student ID:**	832724
PGRS Name:	Giulia Consolandi			
Department:	SEGG	**First Supervisor:**	Alex T. Ford	
Start Date: (or progression date for Prof Doc students)		**October 2016**		
Study Mode and Route:	Part-time ☐ Full-time ☒	MPhil ☐ PhD ☐	MD ☐ Professional Doctorate ☐	

Title of Thesis:	Feeding behaviour as a sublethal endpoint to study the impact of pharmaceuticals on the freshwater amphipod Gammarus pulex
Thesis Word Count: (excluding ancillary data)	46428

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b) Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:	
I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)	
Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	1018A
If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:	
Signed *(PGRS)*:	**Date:** 13/12/19

UPR16 – April 2018